

MOLECULAR ANALYSIS OF GENOMIC VARIABILITY
IN SORGHUM PROTOCLONES

By

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Somaclonal variation was studied in a cell suspension culture and five independent protoplast derived cell suspensions (protoclones) of Sorghum bicolor (L.) Moench cv. NK300 by molecular analyses of their mitochondrial (mt) and nuclear genomes and comparison with those of seedlings. Southern analyses revealed a 14.7 kb EcoRI mtDNA fragment was partially repeated in planta and was hypervariable in all cell suspensions. Each protoclone had a unique hybridization pattern when probed with the 14.7 kb fragment, all were different from the original cell suspension and seedlings.

Genomic arrangement of four mitochondrial protein coding genes was studied by Southern analyses. The mtDNA region containing cytochrome c oxidase subunit I was invariant. The region encoding the alpha subunit of the

F₁-ATPase complex was present as two copies in seedlings and was variable in cell suspensions. Two copies of the gene encoding subunit 6 of the F₀-ATPase complex (atp6) were present in cv. NK300 seedlings. One copy was rearranged in all cell suspensions. Protoclone S63 had a third hybridizing fragment believed to contain a complete atp6 copy. Two arrangements of the region encoding the apocytochrome b subunit of the cytochrome bc₁ complex (cob) were detected in planta. One arrangement was present at substoichiometric levels, and was amplified in cell suspensions. Protoclone S63 had a unique hybridization pattern for the cob region, including loss of the major hybridizing fragment present in planta. Northern analysis of mtRNA revealed a different transcript pattern for cob in S63 than in the seedlings and original cell suspension.

These results identified variability of the mitochondrial genome in tissue culture. Repeated sequences were involved in generating variability. All repeats did not contribute equal levels of rearrangement. A substoichiometric configuration in seedling mtDNA was associated with cob variability in tissue culture. Different arrangements of atp6 were maintained at different levels in cell suspensions. Rearrangement of the cob region was associated with altered transcription.

Southern analyses of sorghum nuclear genome, using maize probes for sucrose synthase genes Sh and Sus,

indicated two non-allelic genes in cv. NK300 seedlings. No variation was detected for these genes in sorghum cell cultures.

CHAPTER 1 INTRODUCTION

Somaclonal Variation

Plant cell and tissue culture systems are now commonly used in basic and applied plant research. A phenomenon associated with plant tissue culture systems, particularly those involving a callus growth phase, is the occurrence of variability in the cultured material. The variation that results from the use of plant tissue culture has been termed "somaclonal variation" (Larkin and Scowcroft 1981). The first reports to recognize the potential of somatic variation for plant improvement concerned tissue culture and regeneration of sugarcane (Heinz and Mee 1971). These were followed by extensive work with potato tissue culture systems, especially regeneration from protoplasts, where a high level of variation is reported in a population of plants derived from the cultivar Russet Burbank (Shepard et al. 1980). The characters that were reported to vary included such useful traits as, maturity date, tuber uniformity and disease resistance. The demonstration that useful variants could be recovered using such methodology

led to many studies of tissue culture associated variability in numerous plant species.

Some studies have tried to elucidate the reasons for the occurrence of somaclonal variation, and changes in chromosome number and structure have been described in many callus cultures and in regenerated plants (reviewed by Lee and Phillips 1988). It must be kept in mind, however, that not all phenotypic variation can be ascribed to such changes in chromosome number. Phenotypic variants have been described with apparently normal chromosome number and karyotype (Gamborg et al. 1977).

Activation of the maize transposable elements Ac and Spm has been reported to occur in regenerated maize plants and is proposed as a possible mechanism for the generation of somaclonal variation (Peschke et al. 1987; Peschke and Phillips 1991). Molecular analyses of somatic variants have been reported in few studies. Maize plants having the cytoplasmic male-sterile (cms) T cytoplasm are susceptible to the fungal pathogen Bipolaris maydis and to the host-specific T-toxin which is produced by the pathogen. Regeneration of plants from calli of cms-T maize resulted in mutants which had reverted to fertility and were resistant to T-toxin. These mutants were recovered with (Gengenbach et al. 1977) and without (Brettell et al. 1980) the imposition of selection pressure for resistance to the toxin. Toxin susceptibility and cms have been shown to be maternally

inherited, and a molecular analysis and comparison of the mitochondrial genomes of fertile regenerants and the sterile progenitor plants demonstrated that a specific mtDNA region in the sterile cytoplasm was responsible for sterility and toxin sensitivity. Detailed molecular analyses of this region of cms-T mtDNA has provided great insight into the events that led to the formation of the novel gene T-urf13 and is a classic demonstration how molecular analyses can aid our understanding of somatic variability.

A number of reports have focussed on a molecular analysis of the rDNA regions of the plant genome. Brettell et al. (1986b) describe quantitative variability for the Nor loci in regenerated triticales plants, and a similar situation has been found in regenerated potato plants where two of twelve plants tested showed quantitative variation for rDNA (Landsmann and Uhrig 1985). Breiman et al. (1987) reported Nor variation in progeny of regenerated wheat plants from the cultivar ND7532, where quantitative and qualitative variation could be detected. In a later report they reevaluated this work (Breiman et al. 1989) and suggest that Nor region variability may have preexisted in the source tissue and is therefore not invariably due to the tissue culture process, and they caution against the use of this region in evaluating the occurrence of somaclonal variation at a molecular level.

Changes in the copy number of genes have been demonstrated in a cell culture system which was exposed to a selection pressure. Cells of alfalfa were selected for resistance to a herbicide which is a selective inhibitor of glutamine synthase, and the resulting tolerant cells exhibited amplification of the number of copies of the glutamine synthase gene (Donn et al. 1984), demonstrating that gene amplification may occur in tissue culture and can be associated with somatic variability.

Point mutations have been demonstrated to be involved in the generation of two somaclonal mutants of the alcohol dehydrogenase (Adh) gene in regenerated maize plants (Brettell et al. 1986a; Dennis et al. 1987). Both mutants were due to single base changes in the coding region of one allele of the gene. It is apparent that many factors can contribute to the generation of somaclonal variation. As pointed out above, the plant mitochondrial genome is amenable to molecular analyses and has proven useful in the characterization of somatic mutants.

Plant Mitochondrial Genome

The mitochondrial genome of plants is large and variable in size, ranging from 200-2400 kb depending on the species examined (reviewed by Lonsdale 1989). Furthermore, while the mitochondrial genome can be described in terms of a "master circle" which contains almost the entire genetic

complement of the mitochondrion, the genome is thought to exist as a population of subgenomic circles which can rearrange via intra- or inter-genomic recombination involving homologous repeat sequences (Lonsdale et al. 1988). Recombination involving repeat sequences is proposed as a mechanism through which mitochondrial genomes may evolve (Small et al. 1987).

While the mitochondrial genome is large and variable, the coding capacity is low based on the total number of polypeptides synthesized in the organelle. Approximately 20 polypeptides encoded by the mitochondrial genome are detected by SDS-PAGE, a number comparable to that found in other eukaryotic systems (Forde et al. 1978). In addition the genes for the 26S, 18S and 5S mt rRNA subunits and a number of tRNAs are present therein. So, at present, it would seem that a large proportion of the mitochondrial genome has no known function.

Mitochondrial Genome Variation

The mitochondrial genome has been particularly well characterized in maize (Lonsdale et al. 1984) and in Brassica species (Palmer 1988) and increasingly so in other plant species such as Sorghum bicolor (Pring et al. 1982; Bailey-Serres et al. 1986a), Oenothera berteriana (Schuster et al. 1987), Triticum aestivum (Hartmann et al. 1989) and

Oryza sativa (Chowdhury et al. 1988, 1990; Kadowaki et al. 1988; Saleh et al. 1990).

The mitochondrial genome of maize has been mapped for N (Lonsdale et al. 1984) and T cytoplasms (Fauron et al. 1989) and differences in gene order, gene copy number and the estimated size of the genome are observed between these cytoplasms (Fauron and Havlik 1989). Differences are also documented in S. bicolor, where the copy number of the gene for subunit 6, atp6 (Pring et al. 1988), and the alpha subunit, atpA (Bailey-Serres et al. 1986a), of the F_1 - F_0 ATPase complex, are found to vary depending on the cytoplasm tested (Bailey-Serres et al. 1986a; Pring et al. 1988). Variation in the restriction endonuclease profile of sorghum mtDNA was reported and used to differentiate groups of cytoplasms (Pring et al. 1982). This quantitative and qualitative variation described for the mitochondrial genome of whole plants also has been described for plant tissue culture systems of species such as tobacco (Grayburn and Bendich 1987), maize (McNay et al. 1984; Chourey et al. 1986b), rice (Chowdhury et al. 1990), wheat (Hartmann et al. 1989) and Brassica campestris (Shirzadegan et al. 1991).

While reports of somaclonal variation exist for sorghum (Bhaskaran et al. 1987; Cai et al. 1990), few reports describe variation at a molecular level in sorghum tissue culture systems (Wilson et al. 1985; Zack and Chourey 1985).

For this reason the work described here examines variation in a number of independent, randomly chosen protoplast derived suspension cultures of S. bicolor cv. NK300 at a molecular level. In particular, since the mitochondrial genome has proven amenable to molecular analyses, most attention was given to studying regions of the mitochondrial genome, some of which code for important mitochondrial proteins. Attention also has been given to analysis of the nuclear genome of the tissue culture material, and two genes which code for sucrose synthase isozymes were studied to determine if they were variable.

CHAPTER II
HYPERVARIABILITY OF A MITOCHONDRIAL DNA REGION
IN SORGHUM PROTOCLONES

Introduction

Somaclonal variation resulting from plant cell and tissue culture is well documented in monocotyledonous and dicotyledonous species (reviewed by Larkin and Scowcroft 1981). These reports of tissue culture variability relate to regenerated plants and their progeny as well as to callus and cell suspension cultures of a number of plant species. The descriptions of somatic variation for callus and suspension cultures include reports of changes in chromosome number and structure (reviewed by Lee and Phillips 1988) and rearrangements of the mitochondrial (mt) genome (reviewed by Lonsdale 1989). The plant mitochondrial genome has been analyzed at a molecular level and detailed reports of tissue culture associated variability of the mitochondrial genome exist (Shirzadegan et al. 1991).

In maize "Black Mexican Sweet" cell suspension cultures quantitative variation, as indicated by altered stoichiometry of certain restriction fragments, is shown to occur (McNay et al. 1984). Such quantitative variation seems to be common in the mitochondrial genomes of plant cell

suspension cultures. A comparison of whole plants and cell suspension cultures from Vicia faba also demonstrates that quantitative differences are found between restriction endonuclease profiles of their mtDNA (Negruk et al. 1986). Grayburn and Bendich (1987) describe differences between two tobacco cell suspension cultures in the abundance of a 4.5 kb mtDNA region cloned from Nicotiana tabacum NT-1. When this region is used in Southern hybridization analyses of mtDNA, from a two year old cell suspension culture of N. tabacum cv. Turkish samsun or a ten year old cell suspension of N. tabacum NT-1, a 70-fold greater hybridization signal is detected in the N. tabacum cv. Turkish samsun culture. No known coding capacity is associated with this region of the mitochondrial genome. The quantitative variability that is detected in the mitochondrial genome of wheat callus cultures has been studied as a function of culture age and reported to be rapidly stabilized after the initial changes occur (Hartmann et al. 1987). Dorfel et al. (1989) described quantitative and qualitative changes, as revealed by restriction fragment and hybridization analyses, in cell suspension cultures of Chenopodium album. They compared two independently derived cell suspension cultures and detected almost identical alterations in the mitochondrial genome of each as compared to the arrangement found in the mitochondrial genome of leaves of C. album, and suggest that rearrangements involve specific regions of the mitochondrial

genome and the variation detected is not a random process involving all regions of the genome. Two mitochondrial genes, coxII and atpA, have been studied by Southern hybridization analyses in this culture system and no variation was reported to occur (Dorfel et al. 1989). A similar suggestion concerning directed rearrangements was proposed by Brears et al. (1989), who found a region of the mitochondrial genome rearranged in sugarbeet plants regenerated from callus of a male-sterile genotype. The rearranged region was identical to the arrangement found in the normal fertile cytoplasm and they suggested that mtDNA has a tendency to undergo specific changes. Extensive variation is documented and well characterized in cell suspension cultures of Brassica campestris (Shirzadegan et al. 1989, 1991). By comparison with the mitochondrial genome arrangement present in B. campestris plants, a two-year old cell suspension culture is shown to have a greatly rearranged mitochondrial genome, the result of inversions and duplications. Some of the observed variation is thought to be due to amplification of substoichiometric levels of rearranged molecules that are present in planta, while other changes may involve de novo rearrangements (Shirzadegan et al. 1989). It is also shown that not all rearranged regions of the mitochondrial genome are stable over time and that continuous quantitative changes involving some of the variable regions occur in long term cell suspension cultures

(Shirzadegan et al. 1991). However, there are mtDNA regions that do not continue to change over time in culture, which is consistent with previous observations (Hartmann et al. 1987). Furthermore, as noted for C. album (Dorfel et al. 1989) and sugarbeet (Brears et al. 1989), it was shown that certain regions of the mitochondrial genome are more likely to be involved in generating variation, as very similar alterations are found in the mtDNA from a number of B. campestris tissue culture lines (Shirzadegan et al. 1991).

These reports of mitochondrial genome variation during tissue culture demonstrate the ability of the mitochondrial genome to change in a short period of time. Such a system may be useful as a means of producing variants which could prove useful in basic studies of mitochondrial genome structure and function.

The objective of this work was to understand some of the molecular events that may be occurring in the tissue culture process. To pursue this, it was decided to analyze the mitochondrial genome of protoplast derived suspension cultures (protoclones) of Sorghum bicolor cv. NK300 (Chourey and Sharpe 1985). Variability in the organization of the mitochondrial genome of different sorghum cytoplasms is known for a number of cytoplasmic male-sterile and fertile lines (Pring et al. 1982; Bailey-Serres et al. 1986a, 1986b). However there are few reports of molecular variation in sorghum as a result of plant tissue culture

(Wilson et al. 1985; Zack and Chourey 1985). The characterization of a 14.7 kb EcoRI fragment of the mitochondrial genome of S. bicolor cv. NK300, which was analyzed in seedlings, a cell suspension culture and five independently derived protoclones is reported, and this region of the mitochondrial genome was found to be hypervariable in the protoclones.

Materials and Methods

Plant Material

Sorghum bicolor cv. NK300 cell suspension culture and protoclones were obtained and grown as described previously (Chourey and Sharpe 1985). Cultures were harvested during logarithmic phase for isolation of mtDNA. Seedlings of S. bicolor cv. NK300 (seed was provided by Dr. F. Miller, Texas A&M University) were grown in the dark for 5-7 days and the mtDNA isolated from the etiolated coleoptiles.

MtDNA Isolation and Analysis

Mitochondrial DNA from cell suspension cultures and seedlings was isolated according to Wilson and Chourey (1984). The mtDNA was digested with restriction enzymes according to the manufacturer's (BRL) instructions. Electrophoresis was performed in 0.8% agarose gels using TAE buffer. Gels were stained with ethidium bromide, $0.5\mu\text{g ml}^{-1}$, for visualization of the DNA.

Cloning mtDNA

Three unique HindIII subclones spanning a previously cloned 14.7 kb EcoRI mitochondrial genome fragment from S. bicolor cv. NK300 were obtained by HindIII digestion of the pBR322 clone harboring the original 14.7 kb EcoRI region and ligation into the HindIII site of pUC 118. The three subclones were referred to as 14.7a (5.3 kb), 14.7b (5.1 kb) and 14.7c (2.8 kb).

Southern Blotting and Hybridization

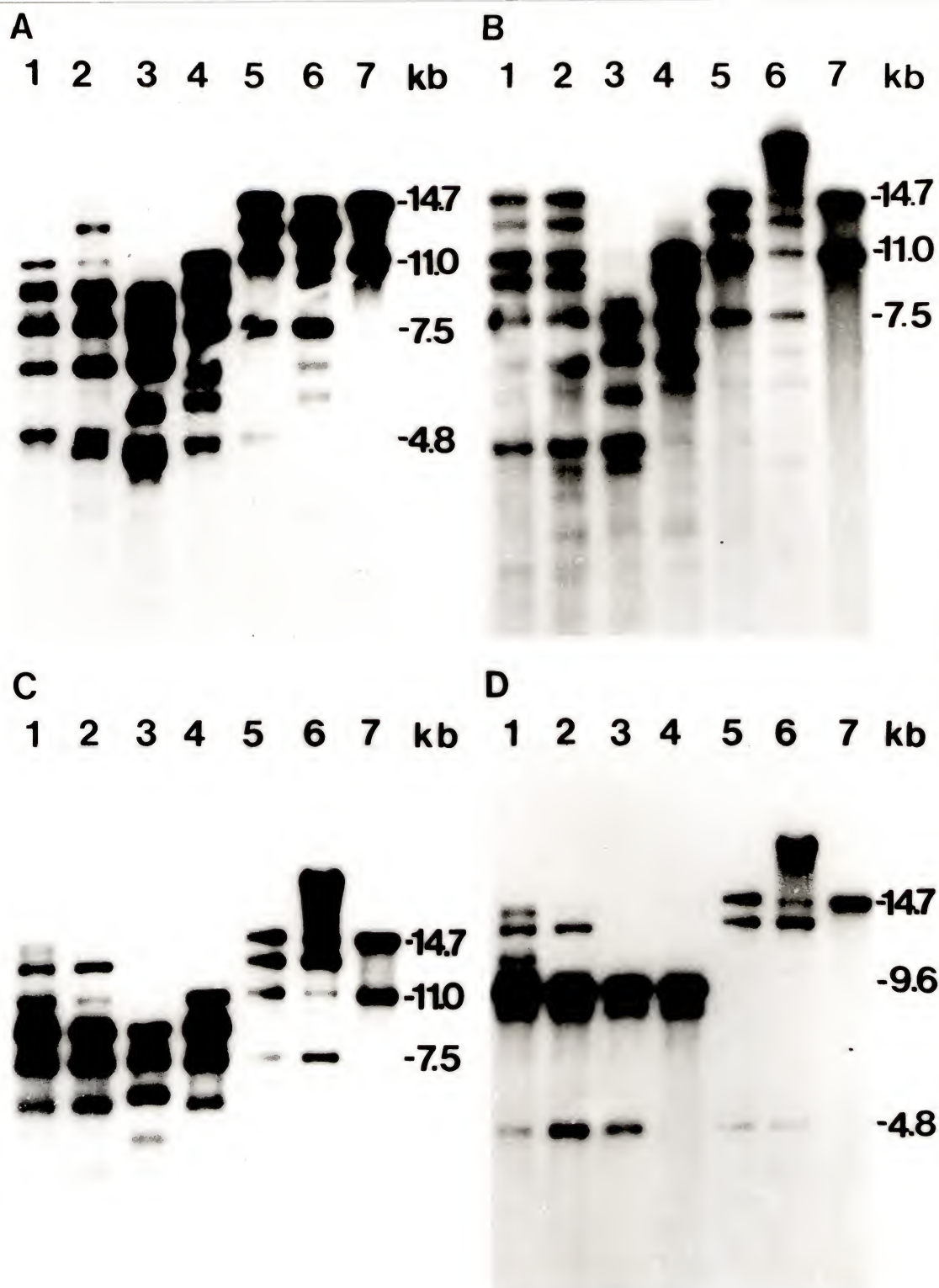
DNA was transferred from agarose gel to Nytran (Schleicher and Schuell) membrane using the procedure of Southern (1975). Membranes were prehybridized for 2-4h at 68°C in 6xSSC, 0.05xBLOTTO (Johnson et al. 1984) and 100µg ml⁻¹ denatured salmon sperm DNA. Hybridization buffer was identical, with the addition of dextran sulphate to 10% w/v, and contained DNA probes labelled with α -³²P-dCTP using the random priming method (Feinberg and Vogelstein 1983). Cloned insert DNA was separated from its vector by agarose gel electrophoresis and recovered using the Geneclean system (BIO 101 Inc.) before labelling. Following hybridization membranes were washed first in 2xSSC, 0.1%SDS at 68°C for 45min and then in 0.3xSSC, 0.1%SDS at 68°C for 45min. Membranes were airdried and autoradiography performed at -70°C using Kodak X-ray film.

Results

Visualization of EcoRI digested mtDNA from S. bicolor cv. NK300 seedlings, cell suspension culture and five randomly selected protocloned revealed that a large proportion of the mitochondrial restriction fragments were unchanged. However, rearrangements and changes in stoichiometry of certain mtDNA fragments in the protocloned were detected. In particular a 14.7 kb EcoRI region was lost in some of the protocloned.

Hybridization of the 14.7 kb EcoRI region to blots of EcoRI digested mtDNA (Fig.2-1.) showed part of this region to be present as a repeat in planta, having homology to EcoRI bands of 14.7 kb and 11.0 kb. When mtDNA from tissue culture cells was examined, additional EcoRI bands were detected at 13.0 kb, 9.6 kb and 7.5 kb. Major rearrangements of the mitochondrial genome were associated with the 14.7 kb EcoRI fragment in the protocloned. Each protocloned had a unique hybridization pattern, all of which were different from both the original cell suspension culture and the seedlings (Fig.2-1.A). Hybridizing fragments ranged in size from 14.7 kb to 4.8 kb. Two protocloned lacked the 14.7 kb EcoRI hybridizing fragment (Fig.2-1.A, lanes 3,4), as expected from the restriction endonuclease patterns. A 7.5 kb EcoRI fragment hybridized to the 14.7 kb region in all the tissue culture lines.

Fig.2-1.A-D. Southern hybridization analysis with cloned mitochondrial fragments A, 14.7kb region; B, subclone 14.7a; C, subclone 14.7b; D, subclone 14.7c, to EcoRI digested mtDNA of protoclonal S50 (1), S51 (2), S63 (3), S181 (4), S262 (5), NK300 cell suspension culture (6), and NK300 seedlings (7).



Hybridization of EcoRI digested mtDNA with each of the HindIII subclones, representing three nonoverlapping subsections of the entire 14.7 kb EcoRI clone are shown in Fig.2-1. Subclone 14.7a and 14.7b each hybridized to both the 14.7 kb and 11.0 kb EcoRI fragments in the seedling mtDNA, while subclone 14.7c hybridized to the 14.7 kb EcoRI fragment alone (Fig.2-1.B-D, lane 7). In tissue culture cells and protoclonal lines each subclone hybridized to multiple EcoRI fragments, emphasizing the uniqueness of each protoclonal line and the original cell culture. Subclone 14.7a (Fig.2-1.B) and subclone 14.7b (Fig.2-1.C) hybridized to the 7.5 kb EcoRI fragment previously found common to all tissue culture lines when the 14.7 kb EcoRI fragment was used as probe (Fig.2-1.A). Use of the complete 14.7 kb EcoRI clone for hybridization with HindIII digested mtDNA detected three bands of 5.3 kb, 5.1 kb and 2.8 kb in the seedling sample (Fig.2-2.A, lane 7), corresponding to each of the HindIII subcloned fragments. Extensive variability was again detected in the tissue culture cells and the protoclonal lines. Hybridization with subclone 14.7a revealed only a single 5.3 kb HindIII band in the seedlings (Fig.2-2.B, lane 7) and indicated that this fragment was a repeat, being present as a 5.3 kb HindIII region in both the 14.7 kb and 11.0 kb EcoRI fragments. Subclone 14.7a also recognized multiple HindIII bands in the tissue culture cells and protoclonal lines (Fig.2-2.B). Two of these HindIII bands, 5.3 kb and 3.0 kb,

were common to all tissue culture material. Use of subclone 14.7b as probe showed hybridization to HindIII bands of 5.1 kb and 2.8 kb in seedlings (Fig.2-2.C, lane 7) and suggested that only part of this region was present on the 11.0 kb EcoRI region which hybridized with this probe (Fig.2-1.C, lane 7). A number of hybridizing fragments were also detected by subclone 14.7b in the different protoclonal lines, but less than the number detected when subclone 14.7a was used as the probe. The 2.8 kb HindIII fragment detected by subclone 14.7b was present in all the samples (Fig.2-2.C). Subclone 14.7c hybridized to a single 2.8 kb HindIII band in the seedlings and all other mtDNA samples (Fig.2-2.D). This region did not seem to be duplicated in planta and was not rearranged in the cell culture line or the protoclonal lines. When mtDNA was digested with XhoI (Fig.2-3.) or with SmaI (Fig.2-4.), and hybridized with each of the four 14.7 kb region probes, a similar situation was found to that revealed by Southern hybridization analyses of EcoRI or HindIII digested mtDNA. In seedlings, two fragments of 16.5 kb and 14.0 kb were detected when XhoI digested mtDNA was hybridized with the complete 14.7 kb EcoRI region (Fig.2-3.A, lane 7). Both fragments were detected when mtDNA was hybridized with each of the three subclones (Fig.2-3.B-D, lane 7), although the 16.5 kb XhoI fragment is recognized to a lesser extent by subclone 14.7c (Fig.2-3.D, lane 7). A 7.7 kb XhoI band was recognized in all the tissue culture samples by the complete

Fig.2-2.A-D. Southern hybridization analysis with cloned mitochondrial fragments A, 14.7kb region; B, subclone 14.7a; C, subclone 14.7b; D, subclone 14.7c, to HindIII digested mtDNA of protoclonal S50 (1), S51 (2), S63 (3), S181 (4), S262 (5), NK300 cell suspension culture (6), and NK300 seedlings (7).

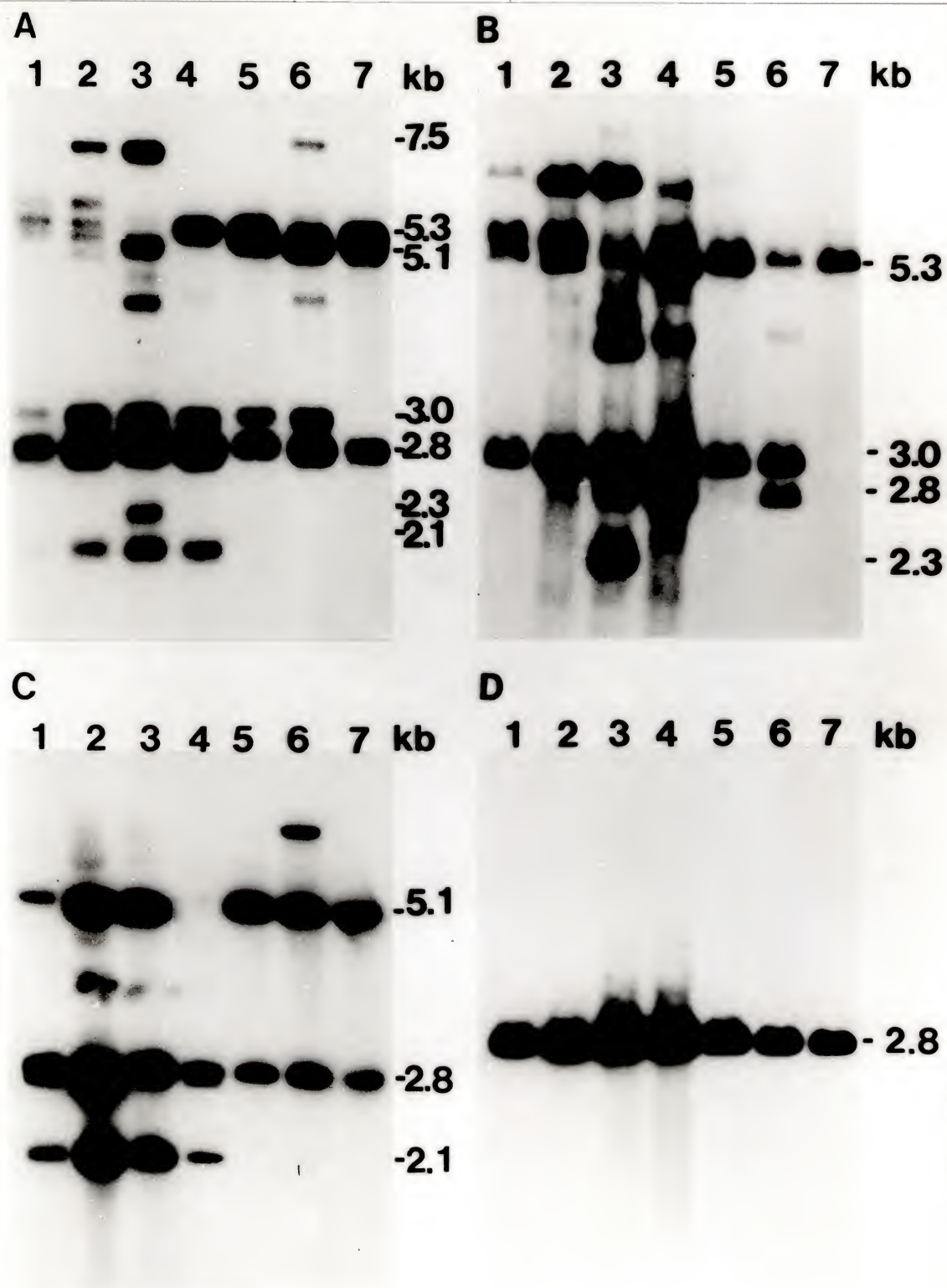


Fig.2-3.A-D. Southern hybridization analysis with cloned mitochondrial fragments A, 14.7kb region; B, subclone 14.7a; C, subclone 14.7b; D, subclone 14.7c, to XhoI digested mtDNA of protoclines S50 (1), S51 (2), S63 (3), S181 (4), S262 (5), NK300 cell suspension culture (6), and NK300 seedlings (7).

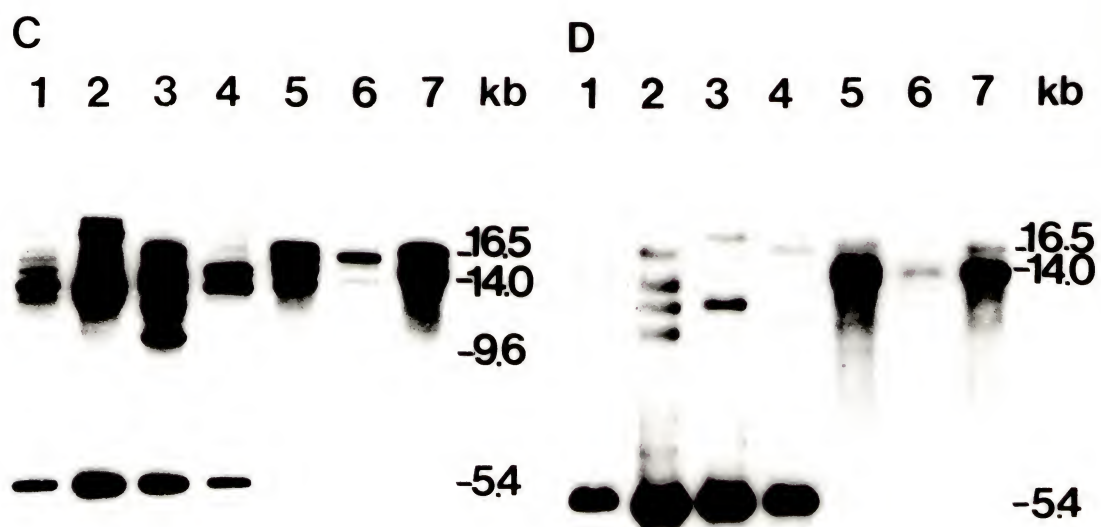
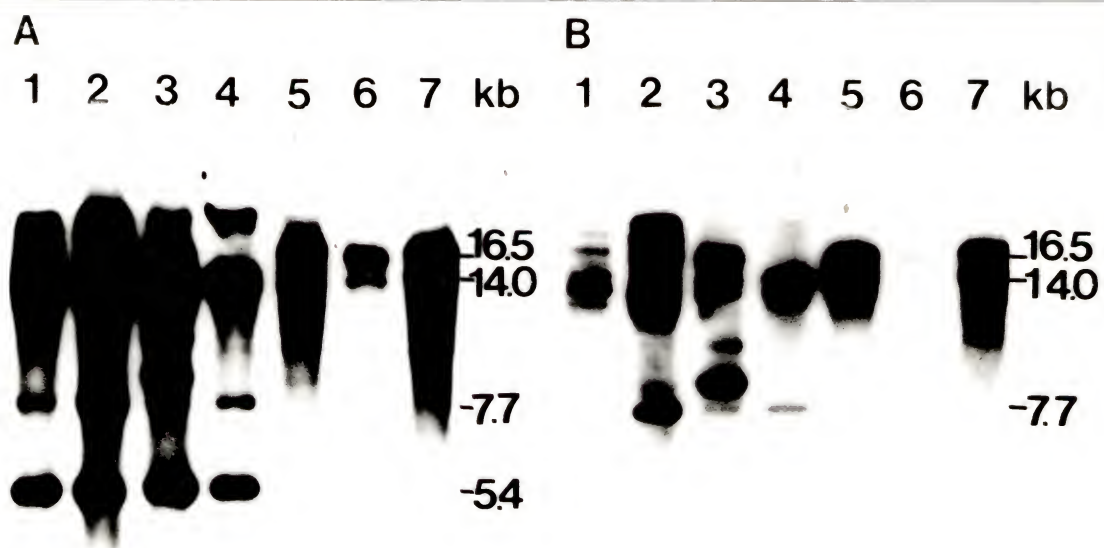
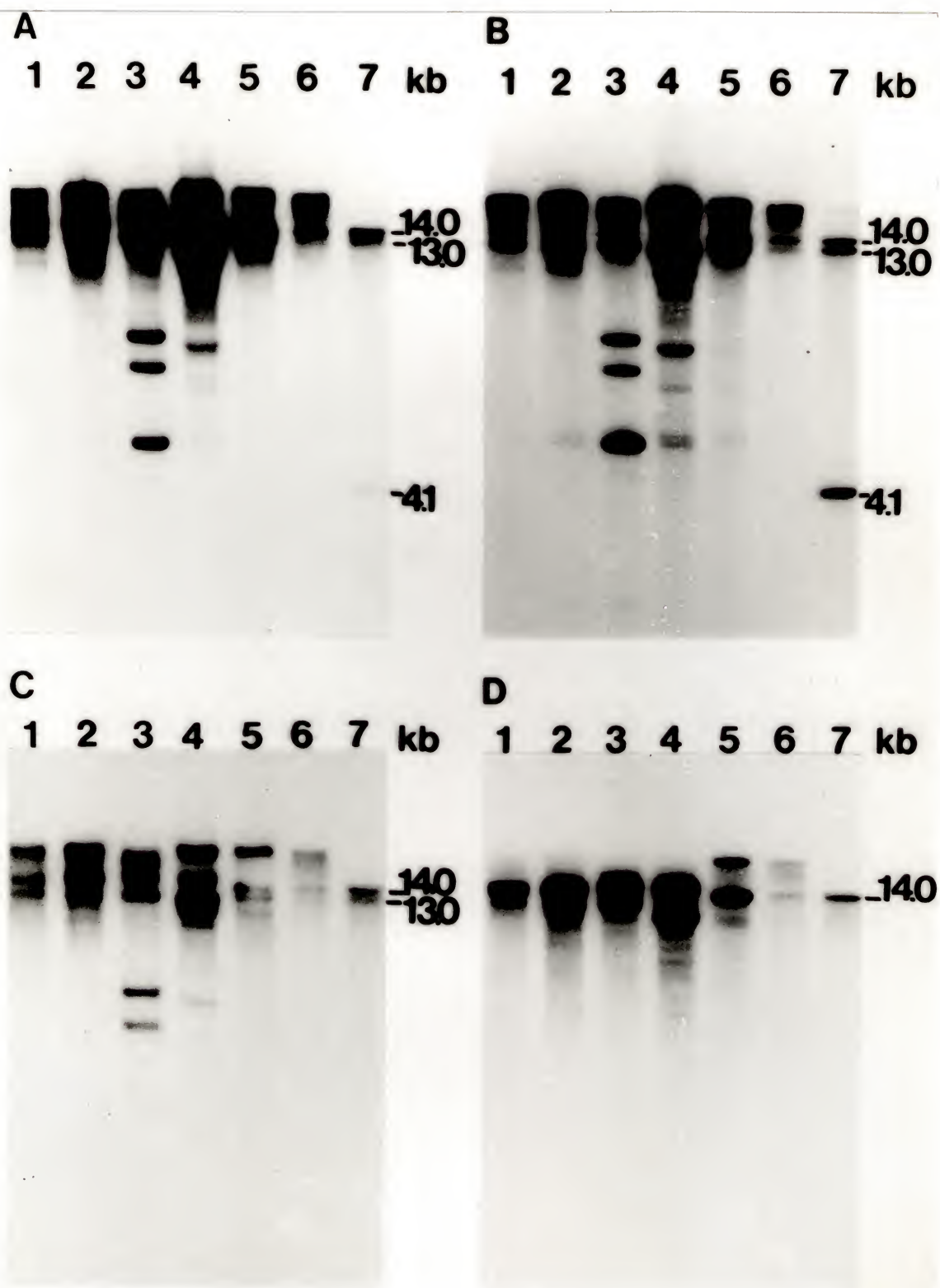


Fig.2-4.A-D. Southern hybridization analysis with cloned mitochondrial fragments A, 14.7kb region; B, subclone 14.7a; C, subclone 14.7b; D, subclone 14.7c, to SmaI digested mtDNA of protoclonal S50 (1), S51 (2), S63 (3), S181 (4), S262 (5), NK300 cell suspension culture (6), and NK300 seedlings (7).



14.7 kb probe and by subclone 14.7a (Fig.2-3.A,B). The stoichiometry of this 7.7 kb fragment varied among the samples, for example it was detected in protoclone S51 at higher levels than in protoclone S63 (Fig.2-3.B, lanes 2,3). A XhoI fragment of 5.4 kb was present in four protoclonal lines when mtDNA was hybridized with the 14.7 kb EcoRI region (Fig.2-3.A, lanes 1-4). This fragment, while not recognized by subclone 14.7a, was detected by subclones 14.7b and 14.7c (Fig.2-3.B-D, lanes 1-4).

The pattern of fragments detected when mtDNA was digested with SmaI and hybridized with the complete 14.7 kb region was less complex in terms of the number of bands seen in the different samples (Fig.2-4) than those seen when each of the other enzymes was used to digest the mtDNA. As noted for the previous restriction enzyme/probe combinations, each of the mtDNA sources did have a unique pattern of hybridizing fragments. Seedling mtDNA had three SmaI bands of 14.0 kb, 13.0 kb and 4.1 kb recognized by the complete 14.7 kb region (Fig.2-4.A, lane 7). The same fragments were detected in seedlings by subclone 14.7a (Fig.2-4.B, lane 7) while subclone 14.7b hybridized to the 14.0 kb and 13.0 kb bands only (Fig.2-4.C, lane 7). A single 14.0 kb SmaI band was detected in seedlings by subclone 14.7c (Fig.2-4.D, lane 7). A 13.0 kb SmaI fragment detected by the complete 14.7 kb probe was common to all the samples (Fig.2-4.A) and also was

detected in each sample by subclones 14.7a and 14.7b (Fig.2-4.B,C).

Discussion

Mitochondrial genome organization of S. bicolor cv. NK300 seedlings, suspension culture cells and protoclonal lines was examined by viewing restriction endonuclease digested mtDNA in ethidium bromide stained gels and by Southern hybridization analyses. Loss or rearrangement as well as changes in the stoichiometry of certain regions of the mitochondrial genome were detected by comparison of the restriction profiles of the different samples. An EcoRI fragment of 14.7 kb was not detected in two of the protoclonal lines. This fragment was further characterized by subcloning, and using the different probes in Southern hybridization analyses of the mtDNA from the different sources.

When the complete 14.7 kb EcoRI fragment and the three HindIII subclones (a,b and c), which spanned the entire 14.7 kb region, were used for hybridization analyses an increase in the number of bands detected was observed in the protoclonal lines relative to the parental seedling and cell suspension mtDNAs (Fig.2-1.-2-4.). Part of the 14.7 kb EcoRI region was present as a repeat in the mitochondrial genome of the seedlings and was very active in generating altered mitochondrial genome organization when cells were subject to

tissue culture, particularly in the five protoclonal lines. This was based on the increase in number of hybridizing fragments in the original suspension culture and all the protoclonal lines, and the loss of some mtDNA regions in certain protoclonal lines. No two protoclonal lines had the same mitochondrial genome arrangement, based on the pattern of hybridizing fragments seen for each. However some hybridizing fragments were common to all tissue culture lines. Variation between some protoclonal lines in stoichiometry of certain common hybridizing fragments confirmed the quantitative variation detected by viewing ethidium bromide stained gels of restriction endonuclease digested mtDNA.

The quantitative and qualitative variation described here for the mitochondrial genome of the sorghum tissue culture material was typical of the many plant tissue culture systems described to date.

The involvement of repeat sequences in generating such mitochondrial genome diversity is well documented (Lonsdale et al. 1988). It has been suggested that there are mitochondrial sequences which are more susceptible to rearrangement and which are primarily responsible for the variation seen in the mitochondrial genome of tissue cultured material (Kemble and Shepard 1984; Brears et al. 1989; Dorfel et al. 1989; Shirzadegan et al. 1989, 1991). Although not all of the 14.7 kb EcoRI region of S. bicolor cv. NK300 seedling mtDNA was present as a repeat in planta

at least 5.3 kb seemed to be repeated, and the repeated mtDNA seemed to be active in generating diversity, typical of the examples referred to above. It is believed that much of the variation seen in the protoclonal was a consequence of the protoplast procedure, since such high levels of variation were not detected for this region of the mitochondrial genome in cell clone suspension cultures (Chourey et al. 1986b) derived from the same parental S. bicolor cv. NK300 cell suspension culture as the protoclonal studied here.

CHAPTER III
GENOMIC VARIABILITY/STABILITY OF MITOCHONDRIAL PROTEIN
CODING GENES, atpA, atp6, coxI AND cob
IN SORGHUM PROTOCLONES

Introduction

A small proportion of the total number of polypeptides present in plant mitochondria are encoded by the mitochondrial (mt) genome. Genes present in plant mitochondria which encode mitochondrial proteins include atpA, atp6, atp9, coxI, coxII, coxIII, cob, those encoding subunits of NADH-dehydrogenase and a number of ribosomal protein genes (reviewed by Newton 1988; Lonsdale 1989).

The atpA gene in plants codes for the alpha subunit of the F₁-ATPase complex, a protein of molecular mass 58 kD in maize (Hack and Leaver 1983), it is a mitochondrial gene in plants and thus differs from the atpA gene of animals which is a nuclear gene (Braun and Levings 1985). The atpA gene is present as a single copy in T-cytoplasm maize, with transcripts of 5000 nt and 2600 nt, and it is predicted to be translated as a protein of 55,178 D (Braun and Levings 1985). In normal male-fertile maize (N cytoplasm) the atpA gene is present as two copies and is associated with one end of a 12 kb direct repeat region of the mitochondrial genome

(Dawson et al. 1986), while it appeared to be present as a single copy gene in the S, C and T cytoplasmic male-sterile (cms) lines (Isaac et al. 1985). However, Braun and Levings (1985) suggest the possibility that there are two distinct copies of atpA in the mitochondrial genome of cms-S maize. This has been confirmed in a number of cms-S lines, where the relative stoichiometry of the different copies is found to vary depending on the lines examined (Wang and Gengenbach 1989).

The atpA gene has been described in a number of plant species, including Nicotiana plumbaginifolia (Chaumont et al. 1988), pea (Morikami and Nakamura 1987), Oenothera (Schuster and Brennicke 1986), radish (Makaroff et al. 1990) and sorghum (Bailey-Serres et al. 1986a). The number of copies of atpA in the mitochondrial genome may vary between species and, as in maize, within lines of the same species. In sorghum either one or two distinct copies are detected, depending on the nuclear-cytoplasmic combination examined (Bailey-Serres et al. 1986a). Further, a 3.9 kb EcoRI fragment seems to be the most common arrangement found in the lines examined, and is the predominant form of the gene in those nuclear-cytoplasmic combinations where only one copy of the gene is detected (Bailey-Serres et al. 1986a). It is also noteworthy that in those lines examined which have milo cytoplasm, regardless of the nuclear background and whether they are sterile or fertile, all have two

distinct copies of the atpA gene on EcoRI fragments of 3.6 kb and 2.3 kb (Bailey-Serres et al. 1986a).

The atpA gene has been studied in a rice tissue culture system, where comparisons have been made between mtDNA of six long term cell suspension cultures and the parental plant which served as source material for the establishment of the cultures. No differences in hybridizing fragments were detected among the different tissues for any of the ten enzyme/probe combinations tested (Chowdhury et al. 1990).

Another mitochondrial gene, atp6, encodes subunit 6 of the F_0 component of the F_1 - F_0 ATPase complex. The atp6 gene has been isolated from cms-T maize, has a complex transcription pattern and is predicted to code for a protein of 31 kD (Dewey et al. 1985). Additionally, the gene contains an internal 122 bp region with homology to the 5' end of the coxII gene in maize, suggesting the possibility for rearrangements of the genome via homologous recombination of these regions. In maize cms-C mitochondria a number of rearrangements are found for the atp6, atp9 and coxII genes. Compared to N and cms-T lines, the atp6 gene in cms-C is chimeric and consists of an atp9 region which forms the 5' flanking region and beginning of the reading frame, a 441 nt region with homology to chloroplast sequences and 804 nt similar to the atp6 gene from cms-T. The gene is apparently present in one copy in cms-C and thought to be functionally active. It is suggested that this or one of the

other two rearranged genes may be responsible for sterility in cms-C lines (Fragoso et al. 1989).

In wheat there are two distinct copies of the atp6 gene which are present on a 1.4 kb repeated region. These genes seem to be actively involved in recombination, in that all four products of the recombination events were detected by Southern hybridization analyses (Bonen and Bird 1988). In soybean mitochondria two distinct copies of the atp6 gene are present, having an identical region of 2.0 kb which extends in the 3' direction but which differ in their 5' regions. Both copies of the gene have homology at their 5' ends to other mitochondrial genes, one to the coxII gene the other to the atp9 gene (Grabau et al. 1988). Makaroff et al. (1989) characterized the atp6 gene in radish mitochondria and compared its arrangement in mitochondria of normal cytoplasm to that present in the Ogura male-sterile cytoplasm. A single copy of the gene is detected in each cytoplasm, although their organization is markedly different. This is true of both the 5' and 3' regions of these genes. No common sequence or structure was found at either the 5' or 3' breakpoints, and it is thought that the rearrangement may have been through homologous recombination involving repeat sequences present near each breakpoint. Mechanisms through which the atp6 gene may contribute to male sterility in Ogura radish have been proposed but definitive proof for its role is lacking.

In sorghum, the atp6 gene is found as a single copy in some cytoplasms and as two distinct copies in others; the copy number is not related to fertility or sterility (Pring et al. 1988). In the IS1112C cytoplasm part of the atp6 gene is present on a repeat sequence and both copies of the gene have a conserved core region (Mullen et al. 1990). Their 5' ends are divergent and result in different size amino extensions, which potentially allow for production of two distinct proteins. It is the larger of the two genes (1146 bp), present on a 5.0 kb BamHI fragment, which is found in all sorghum lines that have been studied. In a tissue culture system, Chowdhury et al. (1990) have examined the mitochondrial genome of long term rice cell suspension cultures and find rearrangement of the atp6 gene in two of the six cell suspension cultures.

The coxI gene, which codes for subunit I of the cytochrome c oxidase, has been studied both in plants (Leaver et al. 1985) and in tissue culture (Chourey et al. 1986b) of sorghum. In most sorghum plant cytoplasms the coxI gene is located on a 4.3 kb EcoRI mtDNA fragment and encodes a 38 kD protein. In the mitochondria of the 9E cytoplasm of sorghum a variant form of the protein is present, being 42 kD in size, and instead of the gene being carried on a 4.3 kb EcoRI fragment it is found on a 10.4 kb EcoRI fragment. Comparison of the two genes shows them to be identical until a position well into the reading frame, after which their 3'

ends diverge. The larger protein is encoded by a gene having a 3' extension which codes for an additional 71 amino acids. This 3' extension is thought to be due to a recombination event, since the regions which flank the common sequence of both genes are located elsewhere in the mitochondrial genome of both cytoplasms (Leaver et al. 1985). The coxI gene in the sorghum cell suspension lines (cv. NK300) is found to be the same form as that which is seen in the seedling mitochondria and is present on a 4.3 kb EcoRI fragment (Chourey et al. 1986b). The coxI gene is well characterized in maize cytoplasms, it is a single copy gene in N, C and T cytoplasms and multi-copy in S cytoplasm (Isaac et al. 1985). Extensive homology exists between the coxI gene in milo cytoplasm of sorghum and that found in maize (Leaver et al. 1985). In a rice tissue culture system the coxI gene is known to be variable in the mitochondria of a number of cell suspension lines when compared to the arrangement found in planta (Chowdhury et al. 1990).

Another plant mitochondrial gene which has been the subject of much study is the gene coding for apocytochrome b, cob. This gene was first isolated from maize (Dawson et al. 1984), where it is reported as a single copy gene in normal cytoplasm (Dawson et al. 1986). In sorghum a single 3.0 kb EcoRI fragment is detected with a heterologous maize probe in Southern hybridization experiments, this is true of all the sorghum lines examined (Bailey-Serres et al. 1986a).

In wheat the cob gene seems to be present as a single copy on a 5.6 kb BamHI fragment, although further analyses using subclones of the original probe indicated a segment of the gene was present elsewhere in the mitochondrial genome. It was suggested that the additional hybridizing region could reflect a low level of another genomic arrangement of the cob gene. The transcription pattern for the wheat cob gene is simple and a single major transcript of 2.4 kb is detected by Northern analysis (Boer et al. 1985), unlike the situation in Oenothera mitochondria where a complex transcript pattern is detected for the cob gene (Schuster and Brennicke 1985).

Besides the four protein coding genes mentioned above, a growing list of mitochondrial genes are being analyzed in a range of plant species (reviewed by Lonsdale 1989). The objective of these studies, in view of the uniqueness of each protoclone, in regard to the 14.7 kb EcoRI mitochondrial region (discussed previously), was to determine a) how mitochondrial protein coding genes were arranged in protoclonal lines of sorghum, and b) if they were rearranged what the effect of rearrangement would have on transcription of the gene.

Materials and Methods

Plant Material

Sorghum bicolor cv. NK300 cell suspension culture and protoclonal lines were obtained and grown as described previously (Chourey and Sharpe 1985). Cultures were harvested during logarithmic phase for isolation of mtDNA. Seedlings of S. bicolor cv. NK300 (seed was provided by Dr. F. Miller, Texas A&M University) were grown in the dark for 5-7 days and the mtDNA isolated from the etiolated coleoptiles.

Isolation and Analysis of mtDNA and mtRNA

Mitochondrial DNA from cell suspension cultures and seedlings was isolated according to Wilson and Chourey (1984). The mtDNA was digested with restriction enzymes according to the manufacturer's (BRL) specifications. Electrophoresis was performed in 0.8% agarose gels using TAE buffer. Gels were stained with ethidium bromide, $0.5\mu\text{g ml}^{-1}$, for visualization of the DNA. For mtRNA preparation, mitochondria were isolated as for mtDNA preparation, lysed in 6M guanidine thiocyanate (Maniatis et al. 1982) and phenol/chloroform extracted prior to treating the nucleic acid with RNase-free DNase. The mtRNA was then recovered by precipitation with ammonium acetate and absolute ethanol. RNA was denatured with glyoxyl and electrophoresed in 1% agarose gels using a 10mM Na_2HPO_4 buffer (pH 7.0).

Mitochondrial Gene Clones

The mitochondrial gene clones used in this study were TA22 which contained a 4.2 kb HindIII mitochondrial fragment carrying the atpA gene from cms-T cytoplasm maize (Braun and Levings 1985), clone 9C2 contained a 240 bp Sau3A fragment from the atp6 gene of sorghum (supplied by Dr. D.R. Pring, University of Florida), clone pKMCOXI which had a 4.3 kb EcoRI insert carrying the coxI gene from sorghum (Bailey-Serres et al. 1986b), and clone pK9ECOB harboring a 3.0 kb EcoRI insert containing the cob gene from sorghum (supplied by Prof. C.J. Leaver, University of Oxford, England).

Southern and Northern Hybridization

DNA was transferred from agarose gel to Nytran (Schleicher and Schuell) membrane using the procedure of Southern (1975). Membranes were prehybridized for 2-4h at 68°C in 6xSSC, 0.05xBLOTTO (Johnson et al. 1984) and 100µg ml⁻¹ denatured salmon sperm DNA. Hybridization buffer was identical, with the addition of dextran sulphate to 10% w/v, and contained DNA probes labelled with α -³²P-dCTP using the random priming method (Feinberg and Vogelstein 1983). Cloned insert DNA was separated from its vector by agarose gel electrophoresis and recovered using the Geneclean system (BIO 101 Inc.) before labelling. Following hybridization for 18h membranes were washed first in 2xSSC, 0.1%SDS at 68°C

for 45min and then in 0.3xSSC, 0.1%SDS at 68°C for 45min. Membranes were airdried and autoradiography performed at -70°C using Kodak X-ray film. RNA was transferred from the gel to Nytran membrane overnight using 20xSSC. Membranes were then baked for 2h before use. Prehybridization solution was as above but with 50% formamide, and incubation was at 42°C for 6h. Hybridization was performed at 42°C for 18h in the same solution with the addition of the labelled probe. Membranes were then treated as above for Southern hybridization analysis.

Results

When mtDNA samples were probed with the insert from pKMCOXI, a 4.3 kb EcoRI fragment containing the coxI gene from milo cytoplasm of sorghum, a single 4.3 kb EcoRI fragment was detected in S. bicolor cv. NK300 seedlings, cell suspension and the five protoclones (Fig.3-1.). No other hybridizing regions were detected in this material.

The atpA gene was also examined by Southern hybridization analyses using the heterologous maize probe TA22, which contains the atpA gene from cms-T cytoplasm, and no major variation was detected between the S. bicolor cv. NK300 cell suspension culture and all the protoclones when mtDNA was digested with EcoRI, BamHI or HindIII (Fig.3-2.). Seedling mtDNA contained two EcoRI fragments which hybridized with TA22 insert mtDNA (Fig.3-2.A, lane7), and no

Fig.3-1. Southern hybridization analysis with pKMCOXI cloned insert to EcoRI digested mtDNA of protoclonal S50 (1), S51 (2), S63 (3), S181 (4), S262 (5), NK300 cell suspension (6), and NK300 seedlings (7).

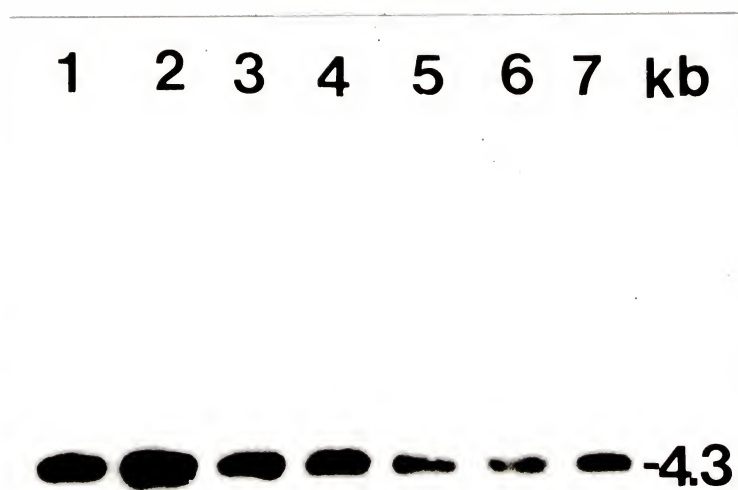
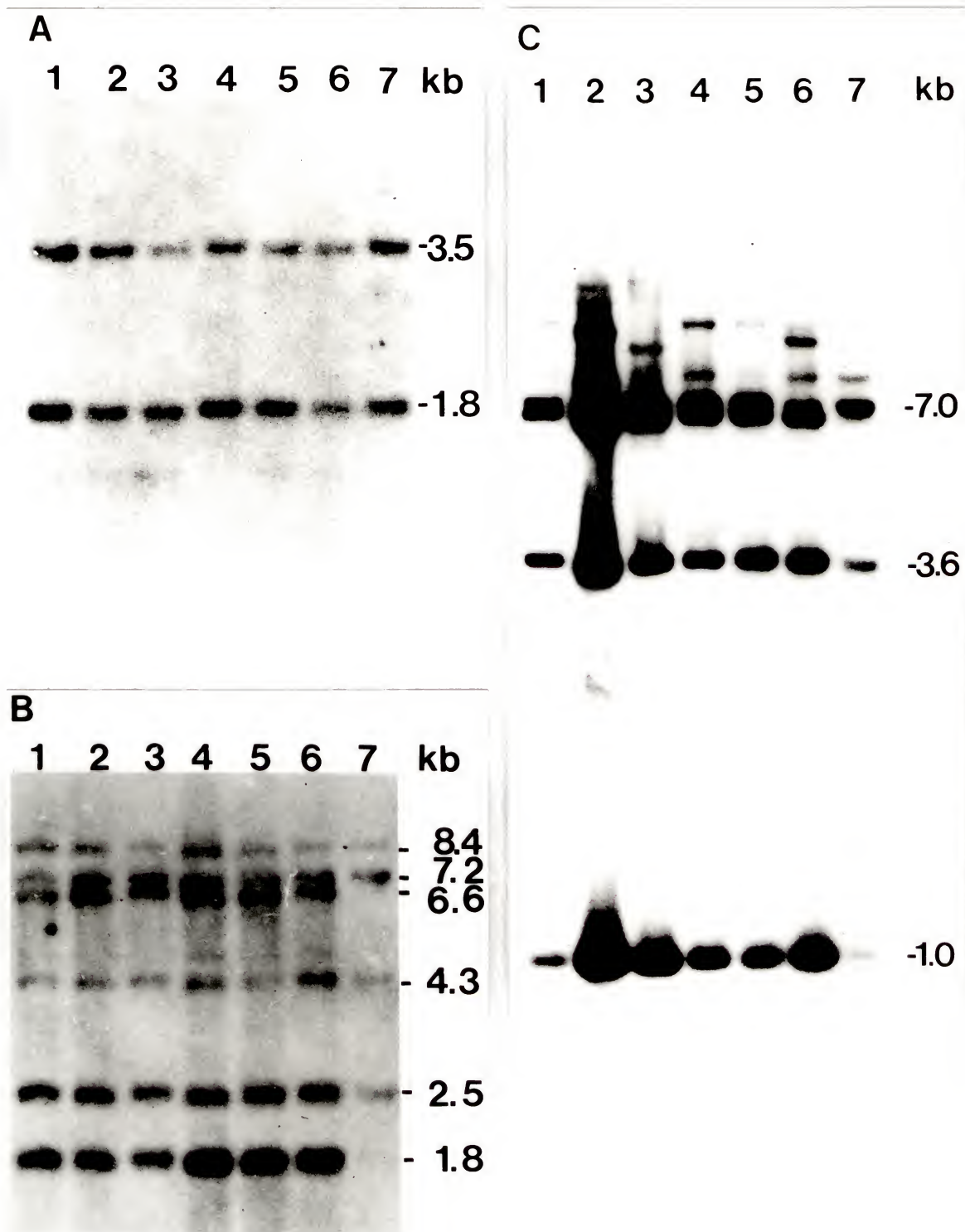


Fig.3-2.A-C. Southern hybridization analysis with TA22 cloned insert to A, EcoRI; B, HindIII; C, BamHI digested mtDNA of protoclonal S50 (1), S51 (2), S63 (3), S181 (4), S262 (5), NK300 cell suspension (6), and NK300 seedlings (7).



variation was evident in the original cell suspension or five protoclones using this enzyme/probe combination; both fragments were present in all the samples (Fig.3-2.A). With HindIII digested mtDNA four fragments hybridized to the TA22 insert in seedling mtDNA (Fig.3-2.B, lane 7). These four regions plus two additional HindIII bands at 6.6 kb and 1.8 kb were present in the original cell suspension culture and the five protoclones. All cell suspension lines had the same pattern of hybridizing regions (Fig.3-2.B). This deviation from the seedling pattern of hybridizing fragments in the cell suspension lines was also evident when mtDNA was digested with BamHI (Fig.3-2.C). While all samples shared most of the hybridizing regions an additional 6.5 kb BamHI fragment was present in all of the cell suspension cultures (Fig.3-2.C).

Two copies of the atp6 gene were detected on BamHI fragments of 5.0 kb and 6.5 kb in S. bicolor cv. NK300 seedling mtDNA when the 9C2 insert was used as probe in Southern hybridization analysis (Fig.3-3.A, lane 7). The cell suspension and all of the protoclones retained the 5.0 kb fragment but instead of the 6.5 kb fragment had a 4.0 kb band in all cell suspension lines examined. Protoclone S63 also contained a unique third band (Fig.3-3.A, lane 3), a 7.0 kb BamHI fragment, which was recognized by the 9C2 cloned insert. The single 3.1 kb fragment detected with the atp6 probe in all samples when mtDNA was digested with EcoRI

(Fig.3-3.B), indicated that a core region common to both copies of the atp6 gene in seedlings was maintained in the cell suspension lines which had the rearranged form of the gene, even in S63 which had three BamHI hybridizing fragments (Fig.3-3.A,B, lane 3). Seedling, cell suspension and protoclone S63 were indistinguishable for atp6 homologous regions when the mtDNA was digested with XbaI and hybridized with the 9C2 insert; all had bands of 4.1 kb and 3.9 kb (Fig.3-4.A, lanes 4-6). When HindIII and BglII were used for digesting mtDNA, seedlings could be distinguished from the two tissue culture lines. All samples had a 1.9 kb HindIII fragment (Fig.3-4.A, lanes 1-3) and a 1.0 kb BglII fragment (Fig.3-4.B), but tissue culture lines had 3.5 kb HindIII and 7.0 kb BglII fragments while the seedlings had 3.0 kb HindIII and 5.5 kb BglII fragments (Fig.3-4.). Besides these differences in arrangement for the mitochondrial regions carrying the atp6 gene in the different sources, it seems that the BamHI regions which hybridized in the two cell suspension lines examined were each maintained at different stoichiometric levels (Fig.3-5., lanes 2-6). Noteworthy was the observation that the 5.0 kb BamHI region was reduced relative to the other BamHI atp6 homologous fragments, and that the 4.0 kb BamHI region was present at the highest level in both of the cell suspensions. Furthermore, each of the three BamHI fragments

Fig.3-3.A,B. Southern hybridization analysis with 9C2 cloned insert to A, BamHI; B, EcoRI digested mtDNA of protoclonal S50 (1), S51 (2), S63 (3), S181 (4), S262 (5), NK300 cell suspension (6), and NK300 seedlings (7).

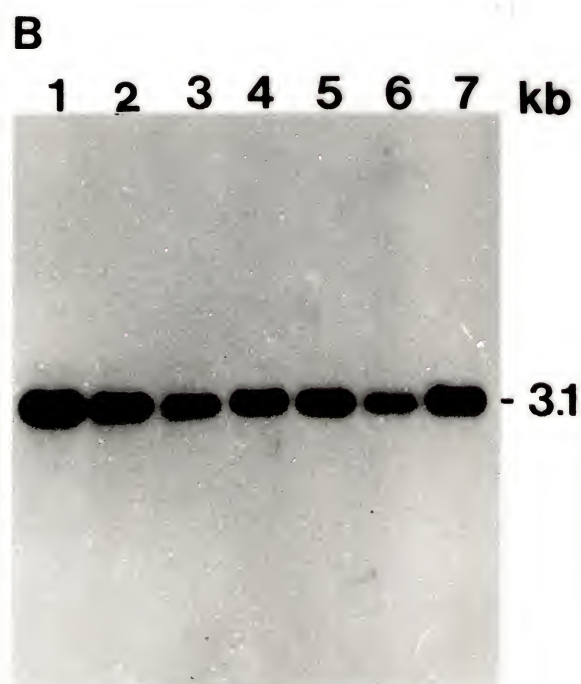
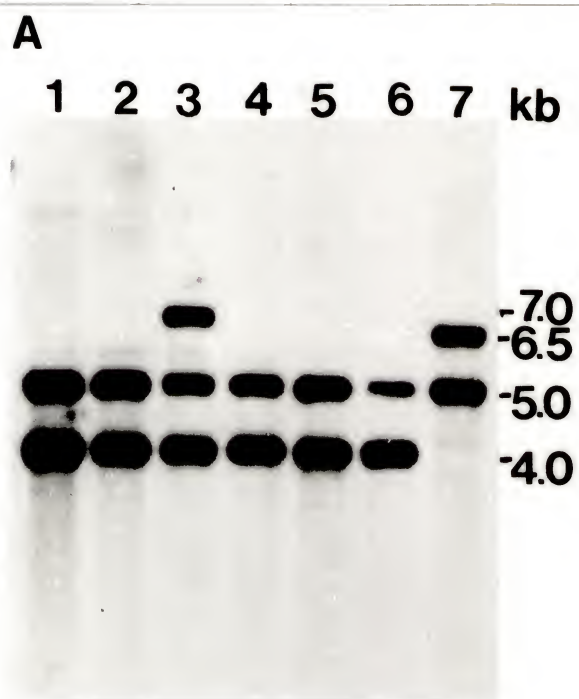


Fig.3-4.A,B. Southern hybridization analysis with 9C2 cloned insert to A, HindIII (1-3), XbaI (4-6); B, BglII digested mtDNA of NK300 seedlings (1&4), NK300 cell suspension (2&5), and protoclone S63 (3&6).

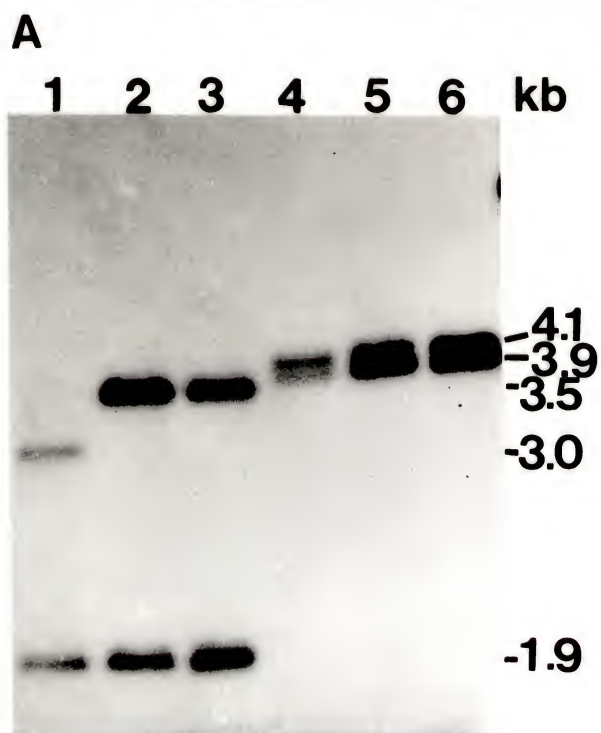
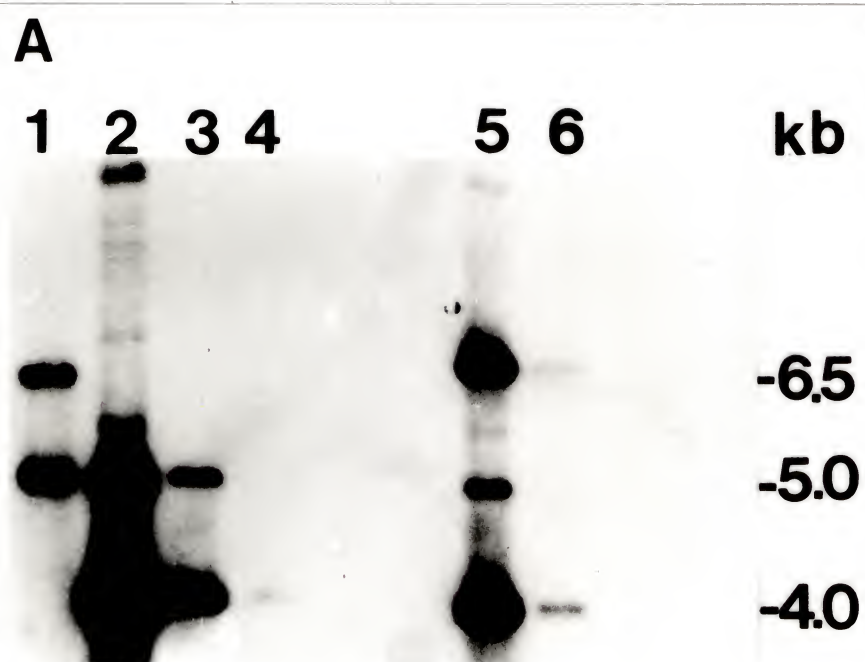


Fig.3-5. Southern hybridization analysis with 9C2 cloned insert to BamHI digested mtDNA of NK300 seedlings (1), ten-fold serial dilutions of NK300 cell suspension mtDNA (2-4), and ten-fold serial dilutions of protoclone S63 mtDNA (5&6).



which hybridized to the atp6 probe in protoclone S63 was present at different levels.

Using a 3.0 kb EcoRI cloned insert, isolated from 9E cytoplasm of sorghum, which contained the cob gene, EcoRI fragments of 3.0 kb and 3.3 kb were detected in S. bicolor cv. NK300 seedling mtDNA by Southern hybridization analyses (Fig.3-6.A, lane 7). The 3.3 kb EcoRI fragment was present in substoichiometric quantities relative to the 3.0 kb EcoRI region in the seedling mitochondrial genome. However, in all tissue culture lines this 3.3 kb fragment was amplified and present in amounts equal to that of the 3.0 kb EcoRI fragment. The 3.0 kb EcoRI fragment was not present in protoclone S63 (Fig.3-6.A, lane 3). Instead two EcoRI fragments were found at 4.0 kb and 4.8 kb. When HindIII digests were hybridized with the cloned insert from pK9ECOB (Fig.3-6.B) a similar difference was noted between the samples; fragments of 2.7 kb and 7.3 kb were found in all cases, while a band at 2.1 kb was present in all but protoclone S63. All tissue culture lines, except protoclone S63, had a 1.9 kb band. The stoichiometry of the 1.9 kb and 7.3 kb fragments was variable among the samples. Protoclone S63 had three extra hybridizing bands at 2.4 kb, 1.7 kb and 1.3 kb (Fig.3-6.B, lane 3). When BamHI digested mtDNA was hybridized with the cloned insert from pK9ECOB, protoclone S63 again had a unique pattern of hybridizing fragments. Another protoclone, S50, was also found to be polymorphic

Fig.3-6.A,B. Southern hybridization analysis with pK9ECOB cloned insert to A, EcoRI; B, HindIII digested mtDNA of protoclonal S50 (1), S51 (2), S63 (3), S181 (4), S262 (5), NK300 cell suspension (6), and NK300 seedlings (7).

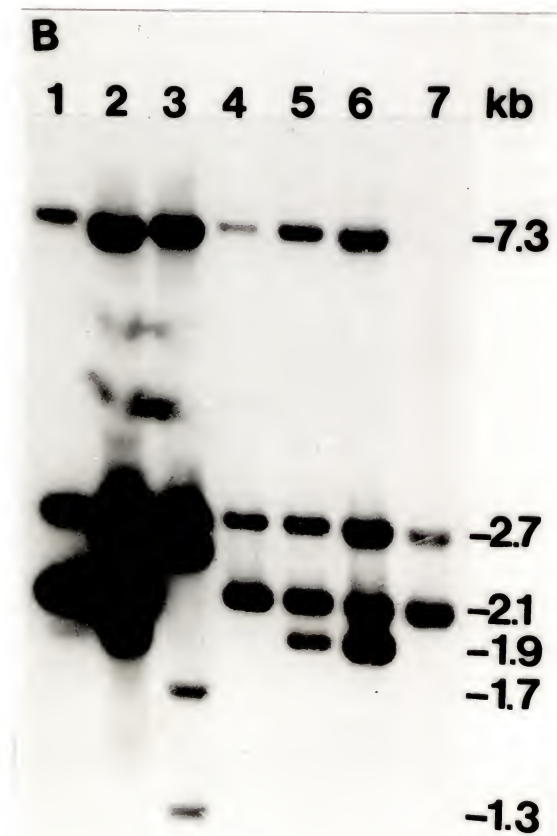
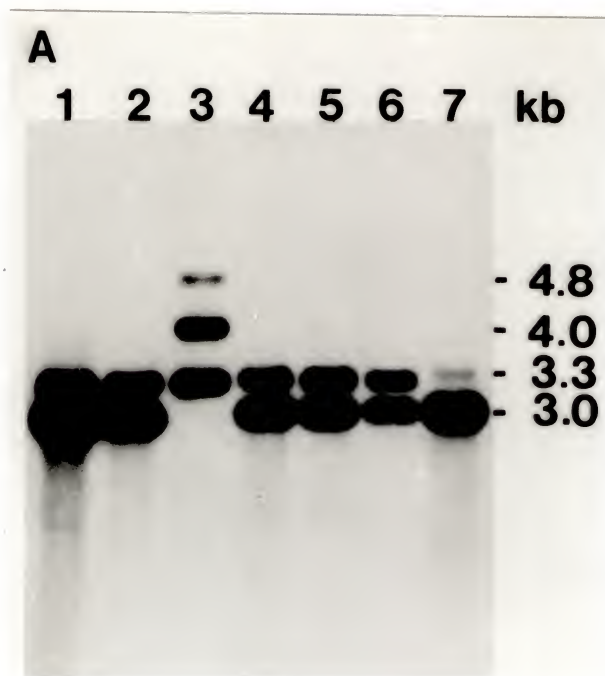
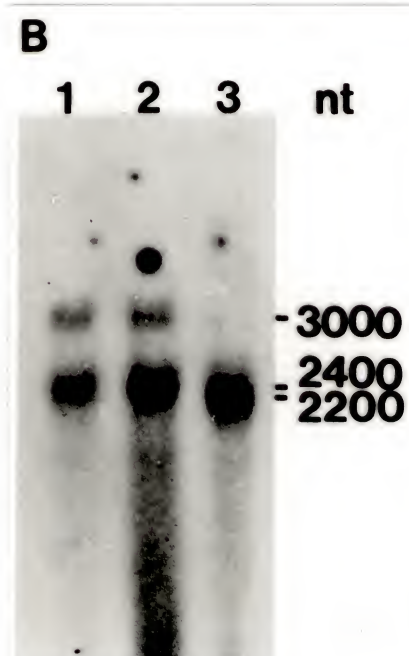
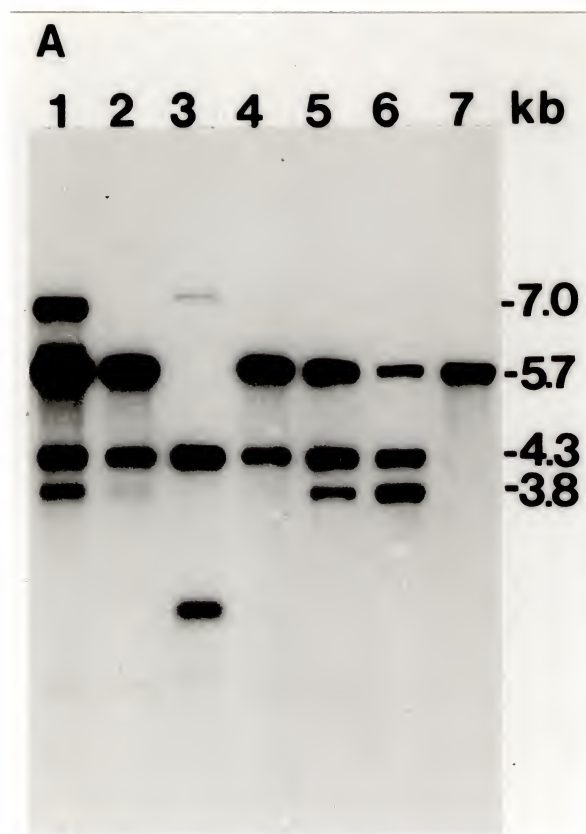


Fig.3-7.A,B. A, Southern hybridization analysis with pK9ECOB cloned insert to BamHI digested mtDNA of protoclonal S50 (1), S51 (2), S63 (3), S181 (4), S262 (5), NK300 cell suspension (6), and NK300 seedlings (7). B, Northern hybridization analysis with pK9ECOB cloned insert to total mtRNA of NK300 seedlings (1), NK300 cell suspension (2), and protoclonal S63 (3).



for the region carrying the cob gene when mtDNA was digested with BamHI (Fig.3-7.A, lane 1). Two unique hybridizing BamHI fragments were found at 6.0 kb and 7.0 kb for protoclone S50. Based on the detected polymorphism in the protoclones, it was decided to examine mitochondrial transcripts by hybridization with pK9ECOB. Comparison of seedlings, cell suspension and protoclone S63 showed that S. bicolor cv. NK300 seedlings and the original cell suspension culture had an identical pattern of transcription (Fig.3-7.B, lanes 1&2), with a major transcript at 2400 nt and a minor transcript at 3000 nt. In protoclone S63 a single major transcript was detected at 2200 nt (Fig.3-7.B, lane 3). It seems that the rearrangements associated with the cob gene in this protoclone resulted in an altered pattern of transcription.

Discussion

Two genomic configurations of the coxI gene in the mitochondrial genome of different sorghum cytoplasms have been reported (Bailey-Serres et al. 1986a). Multiple genomic arrangements are detected for the coxI gene in cms-S maize mitochondria and are believed to be caused by recombination events involving the S plasmids and regions of homology to them found in the 5' region of the coxI gene (Leaver et al. 1985). Evidence of variation for the coxI gene in rice cell suspension cultures has been reported (Chowdhury et al.

1990). No change has been detected in sorghum cell clones and protoclonal lines for the coxI gene arrangement found in seedlings of S. bicolor cv. NK300 (Chourey et al. 1986b). A new configuration was not detected in the material studied here (Fig.3-1.), which was the same as that used in the earlier sorghum tissue culture studies reported by Chourey et al. (1986b).

Little variation was detected for the region carrying the atpA gene in the cell suspension culture or the five protoclonal lines (Fig.3-2.A-C). It seems that following the initial variation found in the cell suspension culture, indicated by the detection of new hybridizing regions in HindIII and BamHI digests, no further changes occurred during the protoplast regeneration of the five protoclonal lines. Variation in genomic location and copy number of the atpA gene has been reported for different cytoplasms in sorghum (Bailey-Serres et al. 1986a). In certain maize cytoplasms the atpA gene is associated with a minimum of four genomic arrangements; in S cytoplasm maize the variation detected is interpreted to be due to existing genomic arrangements, the stoichiometry of which varies depending on the line examined. This difference in stoichiometry of particular gene arrangements is favoured over de novo recombination events as the mechanism which gives rise to such genomic variation in plant mitochondria (Small et al. 1987). Wang and Gengenbach (1989) have also examined the atpA gene in

cms-S maize and describe extensive variation even among progeny from the same maternal parent. They propose that assortment during maternal gamete formation contributes to the differences seen for the atpA gene arrangements among the progeny. In long term rice cell suspensions the atpA gene was found to be stable (Chowdhury et al. 1990).

In this comparison of seedlings and cell suspension lines of sorghum, mitochondrial genome rearrangements were present in the original cell suspension and the protoclines for regions associated with the atp6 gene and the cob gene. The atp6 gene was present in two copies in S. bicolor cv. NK300 seedlings, an arrangement common to a number of different sorghum cytoplasms (Pring et al. 1988). Variation was detected for the atp6 gene upon tissue culture, one copy of the gene was rearranged (Fig.3-3.A), as shown by the absence of the larger 6.5 kb BamHI fragment found in seedlings and the appearance of the 4.0 kb BamHI fragment in all the cell suspension cultures. Further change was noted for one of the protoclines where an extra 7.0 kb BamHI fragment with homology to the atp6 probe was found. Pring et al. (1988) report that at least five repeats exist in mtDNA of certain sorghum cytoplasms, one of which is close to the atp6 gene. A recombinationally active repeat has been reported to be associated with the atp6 gene in wheat where all four products of recombination could be detected (Bonen and Bird 1988). Such an arrangement might be responsible for

the variation detected in the sorghum tissue cultured lines, via intragenomic recombination involving the repeated region associated with atp6. The atp6 gene is also variable in one of six rice suspension culture lines (Chowdhury et al. 1990).

In a previous study of the cob gene in sorghum a single 3.0 kb EcoRI fragment was detected in the five cytoplasms examined, a result similar to that reported here for S. bicolor cv. NK300, although no substoichiometric 3.3 kb fragment was reported previously (Bailey-Serres et al. 1986a). Amplification of this 3.3 kb EcoRI fragment, which has homology to the region carrying the cob gene, to equimolar levels of the 3.0 kb EcoRI fragment in cell suspension cultures (Fig.3-6.A) may have resulted in recombination events giving rise to the rearrangement seen in one of the protoclones.

It is clear from this and other studies that the mitochondrial genome can undergo rearrangement, especially when subject to the tissue culture process. The four mitochondrial protein coding genes examined in this study were expected to be under functional constraints and to remain unaltered. The two regions for which no variation was detected among the tissue culture lines carried coxI or atpA. Fragments carrying these genes have been reported as variant in different sorghum cytoplasms at the whole plant level (Bailey-Serres et al. 1986a). The regions which were

variable in tissue culture carried cob or atp6, and had regions of homology that were present in two unique arrangements in S. bicolor cv. NK300 seedlings, although the level of one region with homology to the pK9ECOB cloned insert was detected at substoichiometric levels in the seedlings. These substoichiometric fragments have been referred to as sublimons (Small et al. 1987). The atp6 gene which is associated with a repetitive region in sorghum may have been rearranged through recombination involving the repeat.

The variability associated with the region carrying the cob gene in the sorghum cell suspension lines could be due to the involvement of the preexisting configuration present in low abundance in the seedlings (Fig.3-6.A, lane 7). Clearly a subgenomic fragment containing a cob homologous region was present in the seedling mtDNA and subsequently amplified in the cell suspension cultures. Furthermore, the maintenance of the atp6 homologous regions at different levels in the cell suspension cultures (Fig.3-5.), suggested that each region was present on unique subgenomic circles which were differentially amplified in the mitochondria and indicates the possible involvement of sublimons in accounting for some of the variation detected. A mechanism for the generation of variation and the evolution of mitochondrial genomes which involves sublimons has been proposed by Small et al. (1987).

Whatever the mechanism involved in generating the variability, it was obvious that regions carrying protein coding genes could be altered in the tissue culture process. Such variation can have an effect on gene expression as shown by the altered transcript pattern detected for the cob gene. Whether this results in altered protein product, as seen in sorghum cytoplasms having a variant form of the coxI gene (Bailey-Serres et al. 1986b), is not known. The rearranged regions of the mitochondrial genome that carry the atp6 or cob genes provide material for further studies on aspects of transcription of these mitochondrial genes in sorghum. This would be facilitated by the development in sorghum of a system for in vitro transcription of mtRNA as recently reported for wheat (Hanic-Joyce and Gray 1991).

The involvement of the nucleus in determining mitochondrial genome structure needs to be considered when evaluating our results. It has been demonstrated that the nuclear genotype is a determining factor for the rate of reversion to fertility in cms-S maize (Laughnan et al. 1981). Small et al. (1988) analyzing cytoplasmic revertants to fertility from a number of CMS-S maize genotypes found they differed in the types of mitochondrial rearrangements which were associated with reversion, and suggested that the nuclear genotype and types of events leading to reversion are linked. Similarly, fertility restoration by the nuclear gene Rf in Phaseolus vulgaris is accompanied by loss of at

least 25 kb of the mitochondrial genome, part of which contains unique sequences (Mackenzie and Chase 1990). Nuclear genotype is also reported as influencing quantitative differences found for mtDNA fragments of alloplasmic cvs of Nicotiana (Hakansson et al. 1990). Since the chromosome number in each of the protoclones and original cell suspension culture are different from each other and from the seedling material (Chourey et al. 1986b) it is possible that the rearrangements detected in the mitochondrial genome of the various cell lines is related to their individual nuclear backgrounds.

CHAPTER IV SUCROSE SYNTHASE GENES IN SORGHUM

Introduction

Somaclonal variation has been analyzed at the molecular level for a number of nuclear genes. The first report of a molecular analysis of nuclear genes to study somaclonal variation is with regenerated potato protoclones (Landsmann and Uhrig 1985), where the 25S rDNA in two of twelve potato regenerants is shown, by Southern hybridization analysis of genomic DNA, to be reduced by 70% relative to the level of the parental material. This reduction in copy number of 25S rDNA is stable through at least one vegetative cycle and seems to have no effect on vigor or morphology of the regenerated potato protoclones (Landsmann and Uhrig 1985). A similar analysis of regenerated triticales plants has shown an 80% reduction in the level of rDNA of one regenerant (Brettell et al. 1986b). Breiman et al. (1987) used Southern hybridization analysis of the nor loci of bread-wheat regenerated plants as a means of evaluating somaclonal variation and demonstrated qualitative and quantitative changes of the nuclear rDNA spacer region in progeny from three regenerated plants from one of three varieties

examined. Analysis of the endosperm proteins, glutenin and gliadin, by SDS-PAGE shows no variation in the same material (Breiman et al. 1987).

A gene encoding alcohol dehydrogenase, Adh1, has been analyzed in maize plants regenerated from callus (Brettell et al. 1986a; Dennis et al. 1987). Two mutants were described, both of which resulted from single base changes in the coding sequence of the Adh gene. One mutant results from an altered amino acid, a glutamic acid having been changed to a valine residue. This results in an electrophoretic variant with reduced mobility compared to the progenitor form (Brettell et al. 1986a). The second variant is a null mutant, due to the replacement of a lysine codon with a nonsense codon in exon 4 (Dennis et al. 1987). These reports show the minor changes that may be associated with tissue culture derived material and the effect they can have.

Despite reports of somaclonal variation in Sorghum bicolor (Bhaskaran et al. 1987; Smith and Bhaskaran 1988; Cai et al. 1990), no molecular analyses of single nuclear genes have been reported to describe somaclonal variation in S. bicolor. The sucrose synthase (SS) genes may provide an interesting system to study in this manner. Maize and sorghum are closely related species and much data exists from analyses of these SS genes in maize. While the gene for SS1 isozyme, Sh, has been shown to hybridize to sorghum DNA

and to be part of a linkage group similar to that in maize (Hulbert et al. 1990), little else is known about these genes at a molecular level in sorghum species. In this report the sucrose synthase genes in S. bicolor cv. NK300 were examined.

The enzyme sucrose synthase (EC 2.4.1.13) catalyzes the reversible conversion of UDP and sucrose to UDPG and fructose, and thus has an important role in cellular metabolism. Sucrose synthase is described for a number of species, including bamboo (Su et al. 1977), wheat (Larsen et al. 1985) and maize (Su and Preiss 1978). It is however best characterized in maize where extensive studies have led to an increased understanding of this important enzyme.

Sucrose synthase exists in maize as two isozymes, SS1 and SS2, encoded by the non-allelic genes, Sh and Sus, respectively. The Sh gene is mapped to the short arm of chromosome nine and is closely linked to the waxy (Wx) and C loci, a linkage group that is also conserved in sorghum (Hulbert et al. 1990). The Sus gene is located on chromosome nine, and has been mapped relative to the Sh locus (McCarty et al. 1986; Gupta et al. 1988). Both isozymes have been compared and shown to be similar for a number of biochemical parameters. They have similar amino acid composition, share antigenic sites and have similar kinetics for sucrose cleavage, although they can be distinguished by protease cleavage patterns (Echt and Chourey 1985).

Both genes have been cloned and characterized from maize (Werr et al. 1985; McCarty et al. 1986; Gupta et al. 1988). While some similarities exist, based on restriction endonuclease mapping and sequence analysis, the genes do diverge and their transcript sizes differ (McCarty et al. 1986; Gupta et al. 1988). Two SS genes are described in wheat and are linked, being mapped to the short arm of group seven chromosomes (Marana et al. 1988).

Of considerable interest is the manner in which these non-allelic genes, Sh and Sus, are expressed. This aspect has been studied in wheat (Marana et al. 1990) and to a much greater extent in maize (Chen and Chourey 1989; Heinlein and Starlinger 1989; Rowland et al. 1989; Rowland and Chourey 1990). In wheat both genes are expressed in the endosperm, the gene encoding the SS1 type protein being highly expressed 18 days after pollination. The gene encoding the SS1 type protein is more abundantly expressed in etiolated leaves and in roots of seven day old wheat plants, it is induced under anaerobic conditions and in response to cold shock, whereas the gene for the SS2 type protein does not respond to such stimuli (Marana et al. 1990). No data were presented on the levels of SS proteins under these conditions. In maize, the Sh and Sus genes encode protein subunits of identical size (87 kD), and the active SS enzyme is a tetramer composed of subunits of SS1 and/or SS2. The composition of the tetrameric protein is found to be tissue

specific. In endosperm, only the homotetramers are detected, in roots and shoots both homotetramers and heterotetramers are present (Chourey et al. 1986a). The lack of heterotetramers in maize endosperm is suggested to be due to either temporal or spatial separation of Sh and Sus gene expression (Chourey et al. 1986a). Confirmation of the temporal and/or spatial separation of Sh and Sus expression in maize endosperm was provided by immunohistological methods (Chen and Chourey 1989) and in situ hybridization analyses (Rowland and Chourey 1990). It is also known in maize that Sh is anaerobically induced at the level of transcription, but a concomitant increase in protein is not observed (McElfresh and Chourey 1988), suggesting posttranscriptional control of Sh expression. It has been demonstrated that the block in expression of Sh is beyond the stage of polyribosomal loading (Taliencio and Chourey 1989). The Sus gene does not appear to be significantly induced by anaerobiosis (McCarty et al. 1986).

Here an analysis of SS genes and SS1 type protein in S. bicolor cv. NK300 is described. The objective was, to determine a) whether there existed two non-allelic SS genes as found in maize and in wheat, and b) to ascertain if somaclonal variants of these genes existed in a group of cell suspension cultures, which included five protoclones. The Southern hybridization analyses to detect polymorphism were performed due to a very high level of variation being

detected in the mitochondrial genome of the same material. Further the SS1 protein was examined by immuno-blot analysis to compare that present in S. bicolor cv. NK300 seedlings to what was found in the cell suspension culture and the protoclones.

Materials and Methods

Plant Material

Seedlings of S. bicolor cv. NK300 (seed was provided by Dr. F. Miller, Texas A&M University) and two Zea mays L. lines, W22 inbred and the inbred genetic stock, sh bz-m4, were grown in flats of vermiculite in the dark for 5-7 days prior to extraction of DNA or protein. Sorghum bicolor cv. NK300 cell suspension culture and protoclones were obtained and grown as previously described (Chourey and Sharpe 1985) as was the maize "Black Mexican Sweet" (BMS) suspension culture (Chourey and Zurawski 1981). Suspension cultures were harvested during logarithmic phase for DNA and protein isolation.

Isolation and Analysis of Genomic DNA

Genomic DNA from seedlings and cell suspension cultures was isolated based on the method of Dellaporta et al. (1983). Briefly, seedling tissue or tissue culture cells were frozen in liquid nitrogen and ground to a fine powder before extraction with buffer, consisting of 100mM Tris (pH

8.0), 100mM NaCl, 1% SDS and 10mM mercaptoethanol.

Extractions were performed at 65°C for 10min, using 15ml buffer per 5g of tissue. Five ml of 5M potassium acetate was then added and the solution incubated on ice for 20min. Following centrifugation at 20,000g for 15min the pellet was resuspended in 50mM Tris (pH 8.0) and extracted with phenol:chloroform, followed by precipitation with 3M sodium acetate and isopropanol. The solution was then centrifuged and the pellet washed with 70% ethanol prior to resuspension in 10mM Tris, 1mM EDTA solution. An RNase A treatment was included, followed by phenol:chloroform extraction and ethanol precipitation, with final resuspension of the nucleic acid in 10mM Tris, 1mM EDTA, pH 7.5.

Ten to 15µg of DNA was digested with the desired restriction enzymes, according to the manufacturer's (BRL) specifications. The digested DNA was then fractionated on a 0.8% agarose gel using TAE buffer, and transferred to Nytran (Schleicher and Schuell) membrane, using the procedure of Southern (1975). Membranes were baked for 2h at 80°C before use in hybridization studies. For hybridization, the membranes were first prehybridized for 2-4h at 68°C in 6xSSC, 0.05% BLOTTO (Johnson et al. 1984) and 100µg ml⁻¹ denatured salmon sperm DNA. Hybridization buffer was the same with dextran sulphate added to 10% w/v and contained the DNA probe of interest. Probes were either pSh9 for the Sh gene or pshD13 for the Sus gene of maize (Gupta et al.

1988) and were labelled with α - ^{32}P -dCTP using the random priming method (Feinberg and Vogelstein 1983). After 18h in hybridization solution the membranes were washed, first in 2xSSC, 0.1% SDS at 68°C for 45min, followed by washing in 0.2xSSC, 0.1% SDS at 68°C for 45min. Membranes were airdried and autoradiography performed at -70°C using Kodak X-ray film.

Isolation and Analysis of Protein

Roots were excised from etiolated seedlings of S. bicolor cv. NK300 and from maize W22 inbred, and 1.0g of tissue was frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. Tris-HCl buffer (10mM, pH 7.2) with 1% mercaptoethanol was added in a 5:1 ratio (v/w) and grinding continued until the buffer was thawed. The homogenate was centrifuged for 10min at 15,000g, the supernatant passed through a glass wool filter and then used for electrophoresis. Samples were electrophoresed on a 4.5-7.0% linear gradient non-denaturing polyacrylamide gel for 16h at 4W constant power. Alternatively samples were denatured with SDS buffer, boiled for 3min, and electrophoresed on an SDS-gel for 4h at 19W constant power.

Following electrophoresis proteins were transferred from the gel to nitrocellulose filter membrane by electroblotting. Transfers were performed for 30min at 4°C in 25mM Tris, 192mM glycine and 20% v/v methanol buffer,

pH 6.5. Following transfer, membranes were placed in blocking solution, 75ml TBS and 2.0g gelatin, for 45min. Blots were then rinsed in TTBS before addition of primary antibody solution containing antisera at a 1/200 dilution. Incubation was for 1h. Antibody solution was recovered and blots rinsed in TTBS. Secondary antibody, goat anti-rabbit alkaline phosphatase was then added to the blots (25 μ l antisera in 75ml of 1% gelatin in TTBS) and incubated for 1h. Blots were then rinsed twice in TTBS for 5min, and once in TBS for 5min. Following this, stain solution was added (7.5mg BCIP dissolved in 500 μ l N-N dimethylformamide added to 50ml of carbonate buffer and 500 μ l NBT solution) and the blot placed in the dark until colour reaction was complete. Blots were removed from stain solution, washed in water, airdried and stored.

Results

Analysis of Genomic DNA

DNA isolated from the different sources was analyzed for the presence of hybridizing fragments with homology to either of the two maize probes for the Sh or Sus genes which code for the SS1 and SS2 isozymes, respectively.

It was shown by Southern analyses that sorghum seedling DNA digested with EcoRI or BamHI (Fig.4-1.A,B, lane 7) and hybridized with pSh9, the Sh cDNA clone, had an EcoRI fragment of 3.2 kb and a BamHI fragment of 9.4 kb. It was

also of interest that the same size bands with homology to pSh9 were detected in the sorghum cell suspension culture and the five protoclonal lines (Fig.4-1.A,B). When the analysis was performed with pshD13 (Fig.4-2.A,B), the Sus cDNA clone, a band of greater than 23 kb was detected when EcoRI digested DNA from sorghum seedlings was examined (Fig.4-2.A, lane 7). With BamHI digested sorghum seedling DNA, fragments of 10.9 kb and 7.0 kb were observed (Fig.4-2.B, lane 7). The hybridizing fragments detected by pshD13 were obviously different to those found when pSh9 was used in hybridization analyses. Further, no variation was detected among the sorghum cell suspension culture and the five protoclonal lines when DNA was hybridized with the pshD13 probe. In addition to the sorghum samples, maize DNA was also included in the analysis, since much is known about these genes in maize plants. In the BMS cell suspension sample, EcoRI fragments of approximately 23.0 kb and 10.9 kb and BamHI fragments of 11.5 kb and 7.8 kb were detected by pSh9, the Sh probe (Fig.4-1.A,B, lane 8). Faint bands were seen in the Sh-deletion strain (sh bz-m4) (Fig.4-1.A,B, lane 9) and were believed to be due to cross hybridization to Sus gene sequences. When pshD13 was used as probe, an EcoRI fragment of greater than 23.0 kb and a BamHI fragment of 7.0 kb were detected in the BMS sample. In sh bz-m4 seedling DNA 18.0 kb EcoRI and 7.0 kb BamHI fragments were detected with pshD13

Fig.4-1. Southern hybridization analysis with SS1 cDNA probe to A, EcoRI; B, BamHI, digested genomic DNA of protoclonal S50 (1), S51 (2), S63 (3), S181 (4), S262 (5), NK300 cell suspension (6), NK300 seedling (7), Black Mexican Sweet cell suspension (8), and sh bz-m4 seedling (9).

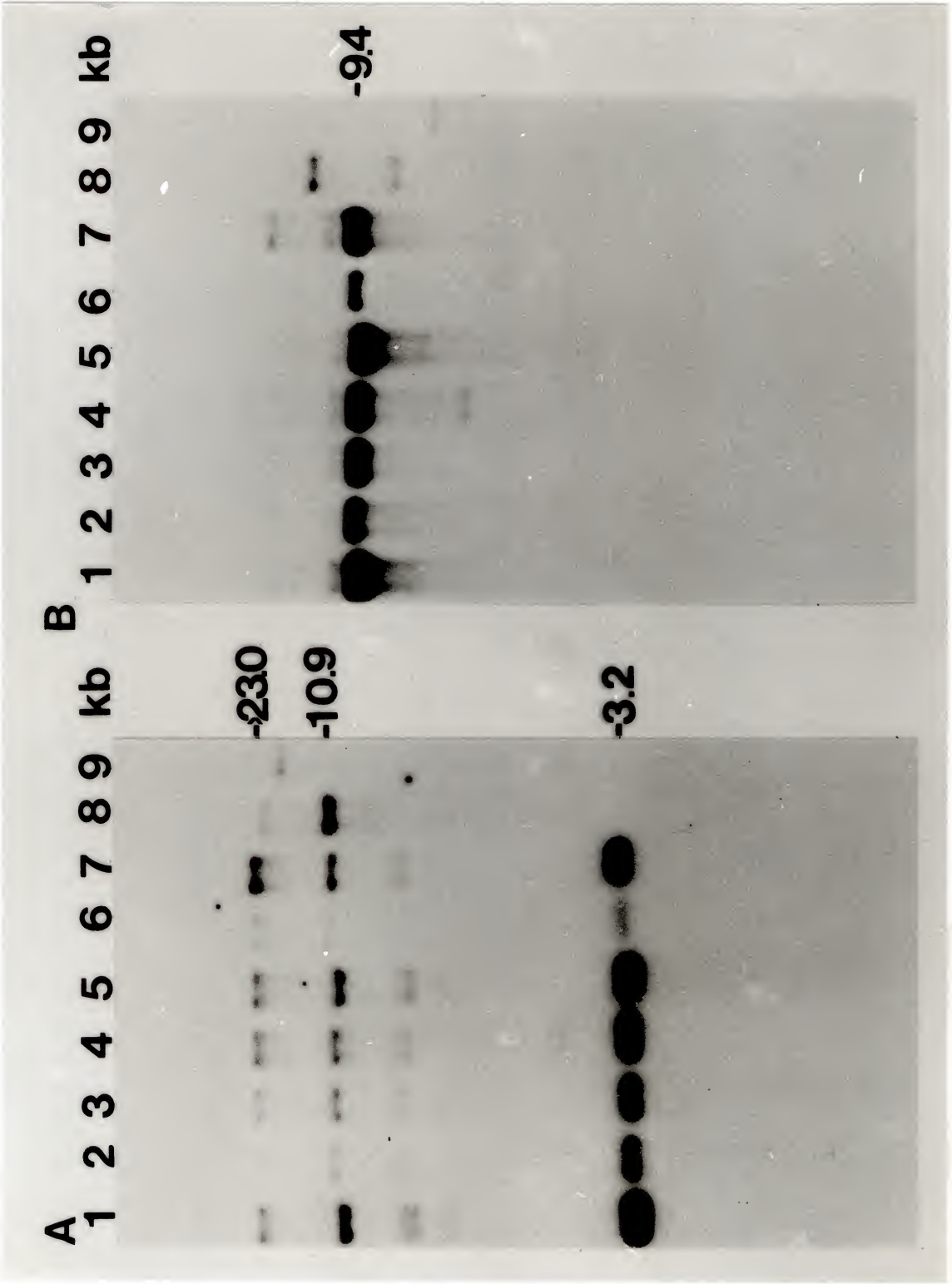
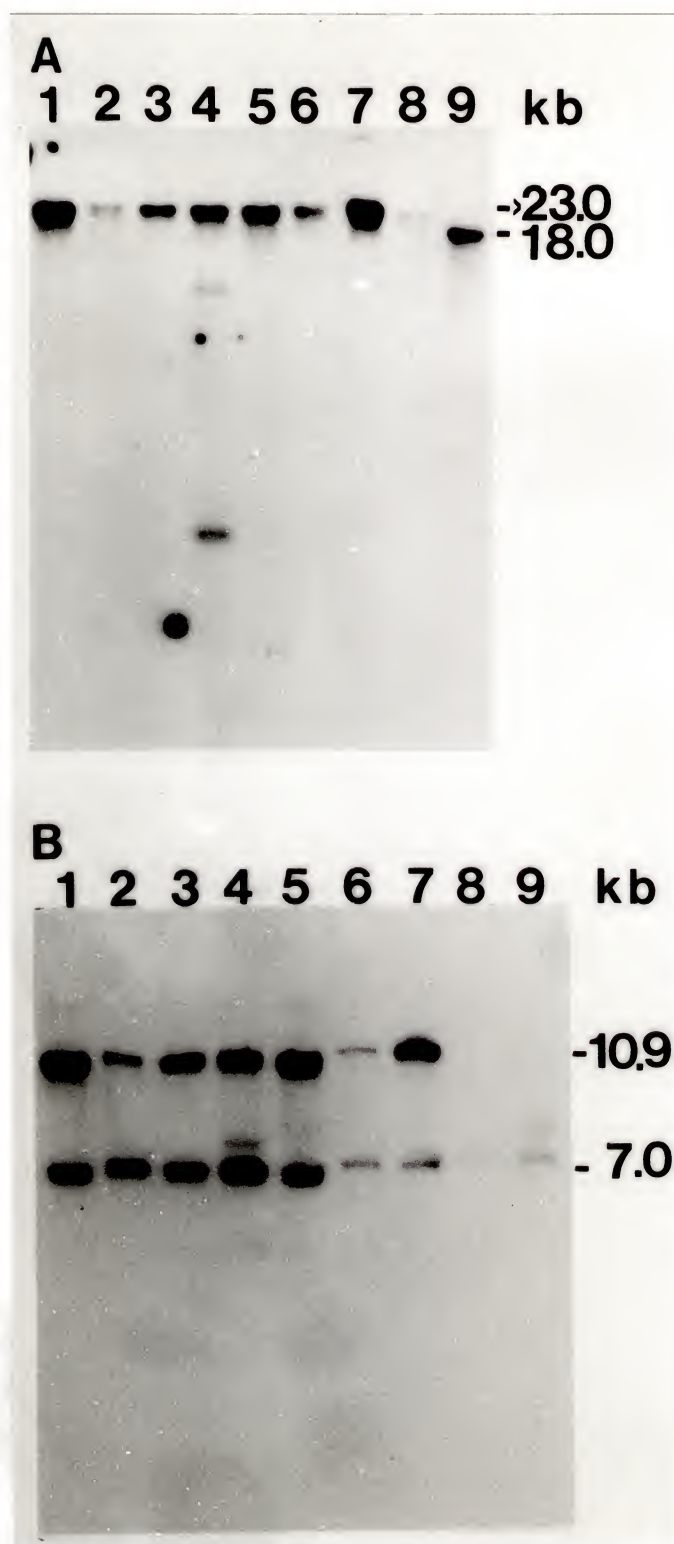


Fig.4-2.A,B. Southern hybridization analysis with SS2 cDNA probe to A, EcoRI; B, BamHI, digested genomic DNA of protoclones S50 (1), S51 (2), S63 (3), S181 (4), S262 (5), NK300 cell suspension (6), NK300 seedling (7), Black Mexican Sweet cell suspension (8), and sh bz-m4 seedling (9).



and compare to previous reports for this genotype (Chourey et al. 1988).

Clearly, the fragments which hybridized with sorghum DNA samples were not the same size as those detected in the genomic DNA from the maize tissues examined here (Fig.4-1. and 4-2.).

Analysis of SS1 Protein

In addition to DNA analysis, SS1 protein was examined. Western blot analyses (Fig.4-3.) revealed that no differences in the protein reacting with the heterologous polyclonal antisera, produced against maize SS1, were detected among all the sorghum samples. This was the case with both native (Fig.4-3.A) and denaturing conditions (Fig.4-3.B). It was found that SS1 was the major sucrose synthase isozyme in sorghum cell suspension cultures since fewer heterotetramers were detected. It was also noteworthy that the native form of the SS1 homotetramer in sorghum was the fastest migrating of all the bands, unlike the case found in maize. Additionally, it was shown that the native form of the SS tetramers in sorghum migrated at a different position in the gel to those of maize (Fig.4-3.A). On denaturing gels, all sorghum samples had a single band reacting with SS1 polyclonal antisera. The sorghum SS1 type protein subunit was the same size as the SS1 subunit from maize (Fig.4-3.B).

Fig.4-3.A,B. Western blot analysis with SS1 polyclonal antisera to A, native gel; B, SDS denaturing gel of protein extracted from Pioneer root (1), NK300 root (2), Black Mexican Sweet cell suspension (3), NK300 cell suspension (4), protoclonal S262 (5), S181 (6), S63 (7), S51 (8), S50 (9), and Pioneer root (10). Anodal end is at the bottom of the figure.

A**1 2 3 4 5 6 7 8 9 10****B****1 2 3 4 5 6 7 8 9 10**

Discussion

Genomic Southern hybridization data showed that two unique sucrose synthase loci, referred to as Sus1 and Sus2, homologous to the maize Sh and Sus genes, existed in sorghum seedlings and cell suspension cultures. The fragments detected using pSh9 when genomic DNA was digested with EcoRI or BamHI, were distinct from those detected by pshD13. These loci were also distinct from their analogous genes in maize, as seen by the difference detected in fragment sizes between the sorghum and maize samples. While it remains to be seen whether these two loci are linked, as they are in maize (McCarty et al. 1986; Gupta et al. 1988) and wheat (Marana et al. 1988), a linkage group which includes the Sh gene in maize is conserved in sorghum seedlings (Hulbert et al. 1990). Analysis of genomic DNA from sorghum cell suspension and protoclones did not reveal polymorphism for these loci with the two cDNA probes and enzyme combinations examined. This was surprising in view of the variability in chromosome number of these cell suspension cultures (Chourey et al. 1986b) and high levels of mitochondrial genome variability and indicated how certain regions of the genome could remain unchanged despite hypervariability in other parts of the plant genome.

Of interest, was the immuno-blot analysis of the Sus1 encoded protein using polyclonal antisera. No variation was

detected among the cell lines and seedlings, either on native or SDS denaturing gels. From analysis with native gels it was evident that the SS1 type protein was abundantly produced in cell suspension cultures. Based on the five isozymes and the similar size of the denatured protein, it could be envisaged that the active protein was a tetramer, as found for maize. Comparison between maize and sorghum root samples on native gels suggested that the SS1 type protein was more abundant in sorghum roots than the SS2 type, since the prominent band seemed to be the SS1 type homotetramer and not the heterotetramers as seen in the maize root samples. Another difference was that the SS1 type homotetramer was the most anodal migrating isozyme in sorghum seedling and cell suspension cultures, unlike the situation in maize where the SS1 homotetramer is the least anodal form. On SDS denaturing gel it was obvious that the SS1 type subunit in sorghum was the same relative molecular weight as SS1 from maize.

Recently, further analyses of the Sus1 and Sus2 genes and the proteins they encode have been reported in sorghum (Chourey et al. 1991). It has been demonstrated that the sorghum sucrose synthases have much in common with their counterparts in maize, but also possess unique attributes. As found in maize, the sorghum Sus2 transcript is slightly larger than the Sus1 transcript. However, unlike maize, there is no difference in protein subunit size in sorghum.

Both Sus1 and Sus2 in sorghum respond to anaerobiosis by producing elevated levels of transcript, but no increase in sucrose synthase protein level occurs under anaerobic stress in sorghum. A final difference noted by Chourey et al.

(1991) in sorghum is the tissue specificity of Sus1 and Sus2 expression. On native gels five isozyme bands are detected for sorghum endosperm samples, suggesting that both genes are simultaneously expressed in endosperm cells, a situation different from maize endosperm where spatial separation of Sh and Sus gene expression occurs (Chen and Chourey 1989).

The data presented here, and that reported by Chourey et al. (1991), provide evidence for the presence of two sucrose synthase isozymes encoded by non-allelic genes in sorghum. As determined by analysis of the cell suspension culture and the protoclones these two nuclear genes, Sus1 and Sus2, are apparently stable in this tissue culture system.

CHAPTER V CONCLUSION

Somaclonal variation continues to be used as a means of generating novel plant material for use in breeding programs, particularly those aimed at improving disease resistance (Heath-Pagliuso and Rappaport 1990; Wenzel and Foroughi-Wehr 1990). The cause of somaclonal variation remains a relative mystery, although the genetic consequences are being increasingly revealed and described.

Here a description of the variation detected in the mitochondrial genome of Sorghum bicolor cv. NK300 cell suspension culture and five independently derived protoclones was presented. Based on this and other studies of mitochondrial variation, the mitochondrial genome may be considered as consisting of three distinct types of regions: those that were highly variable under the tissue culture stress; those that varied in a less dramatic manner; and those that were stable.

Each of the above categories were exemplified in the analyses of the 14.7 kb EcoRI region, and the four mitochondrial regions carrying the protein coding genes atpA, atp6, coxI and cob, in the mitochondrial genome of S. bicolor cv. NK300 tissue cultured material.

Part of the 14.7 kb EcoRI mtDNA region was present as a repeat in planta. This could account for the very high level of rearrangement associated with this mtDNA region in the protoclones. Since all the cell suspension cultures showed no obvious deleterious effects, the intact 14.7 kb EcoRI region was apparently not important for cell viability. It should be noted however, that with all the enzymes used for digestion of the mtDNA, a single conserved fragment was always present in all the cell suspensions when hybridized to the 14.7 kb EcoRI region. At present, the significance of this observation is not known.

While the rearrangements detected for the 14.7 kb EcoRI region appeared to be stable over a prolonged culture period, this does not necessarily have to be true of all regions of the mitochondrial genome, as demonstrated recently for certain areas of the Brassica campestris mitochondrial genome in cell cultures (Shirzadegan et al. 1991). The true basis for stability of a particular mtDNA region versus the high level of variation of another mtDNA region is presently a matter of conjecture.

Southern hybridization analyses of protein coding regions of the mitochondrial genome of S. bicolor cv. NK300 cell suspensions demonstrated that while variation was possible in these regions the extent of the rearrangements was less than that seen for the 14.7 kb region. Most protoclones had the same pattern of rearrangement when such

variation was present. This pattern of common rearrangements was indicative of a certain region being more prone to alterations as has been suggested in a number of plant systems (Brears et al. 1989; Dorfel et al. 1989), or of particular subgenomic molecules being selected for and preferentially replicated. The rearranged regions of the genome which contained mitochondrial protein coding genes were, in all cases, associated with a region of repetitive DNA. In one case, involving the region carrying the cob gene, one of the repeats was present as a sublimon in planta. Following the rearrangement of one copy of the atp6 gene in cell suspensions, each genomic arrangement was present at different levels. These results supported and demonstrated the importance of repetitive DNA and sublimons in determining the mitochondrial genome arrangement, as proposed by Lonsdale et al. (1988) and Small et al. (1989). The effects of such perturbations of the mitochondrial genome were observed to have a possible effect on transcription of the cob gene, and demonstrated the ability to recover from the tissue culture system possible interesting plant mitochondrial mutants, useful for basic studies on aspects of mitochondrial gene regulation. Evidence was also presented using a region carrying the coxI gene as hybridization probe, that a 4.3 kb EcoRI region was recognized in planta and no deviation from this hybridization pattern was detected among all the cell

cultures. This was true over a prolonged culture period, which is known to contribute to increased variability in nuclear genome of cell suspension cultures.

As mentioned earlier, an in vitro mitochondrial transcription system has been developed recently for wheat (Hanic-Joyce and Gray 1991). The development of such a system for sorghum, in combination with the isolation of the altered regions which carry mitochondrial protein coding genes from the protoclones, could provide a means of studying factors important in mitochondrial gene regulation. This could lead to an increased understanding of mitochondrial gene regulation in a manner that has been demonstrated in yeast for the cob gene (reviewed by Costanzo and Fox 1990).

The importance of the effects of the nuclear genome of each cell line, which are known to differ from the usual chromosome content of the seedlings (Chourey et al. 1986b), on the alterations detected in the mitochondrial genome of each cell suspension cannot be overlooked. The effect of the nucleus has recently been shown to be a factor in mitochondrial genome arrangement (Hakansson et al 1990) and mitochondrial gene expression (Cooper et al. 1990) of plants. While the effects of the nuclear genome on mitochondrial genome structure were not studied here, nuclear genes encoding sucrose synthase were analysed. The sucrose synthase genes were found to be present as two non-

allelic genes in the sorghum seedling material, as is the situation in maize and wheat. No polymorphism was detected, within the limits of resolution, in the tissue culture lines. This lack of variation was confirmed by Western blot analyses of the SS1 type protein, where the protein banding pattern was identical in all sorghum tissue culture samples.

It was shown by this work that there is a great ability of the mitochondrial genome to vary in tissue culture and result in the generation of new configurations containing mitochondrial protein coding genes as well as altered regions not known to be involved in gene expression. It is believed such variation, while confirming the phenomenon of somaclonal variation at a molecular level, will also provide a useful source of plant material for basic studies of mitochondrial gene regulation. It was also interesting that, despite the obvious variation in mitochondrial genome of all the cell suspensions, the genes for sucrose synthase were not rearranged, at least based on the enzyme/probe combinations used.

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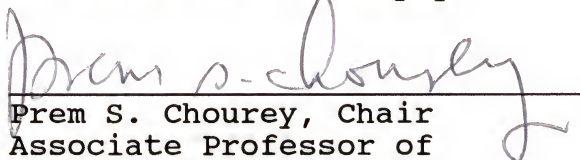
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